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ISBN 978-90-367-9881-5
ISBN 978-90-367-9880-8

The printing of this thesis was financially supported by: Rijksuniversiteit Groningen.

Cover design: Hessel Hemstra

Printing: Ridderprint BV, the Netherlands

rijksuniversiteit groningen

Clinical, biochemical and genetic aspects of glycogen storage disease type III

Proefschrift

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. E. Sterken
en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 14 juni 2017 om 14.30 uur

door

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geboren op 20 september 1984
te Groningen

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Chapter 1

General introduction & outline of the thesis

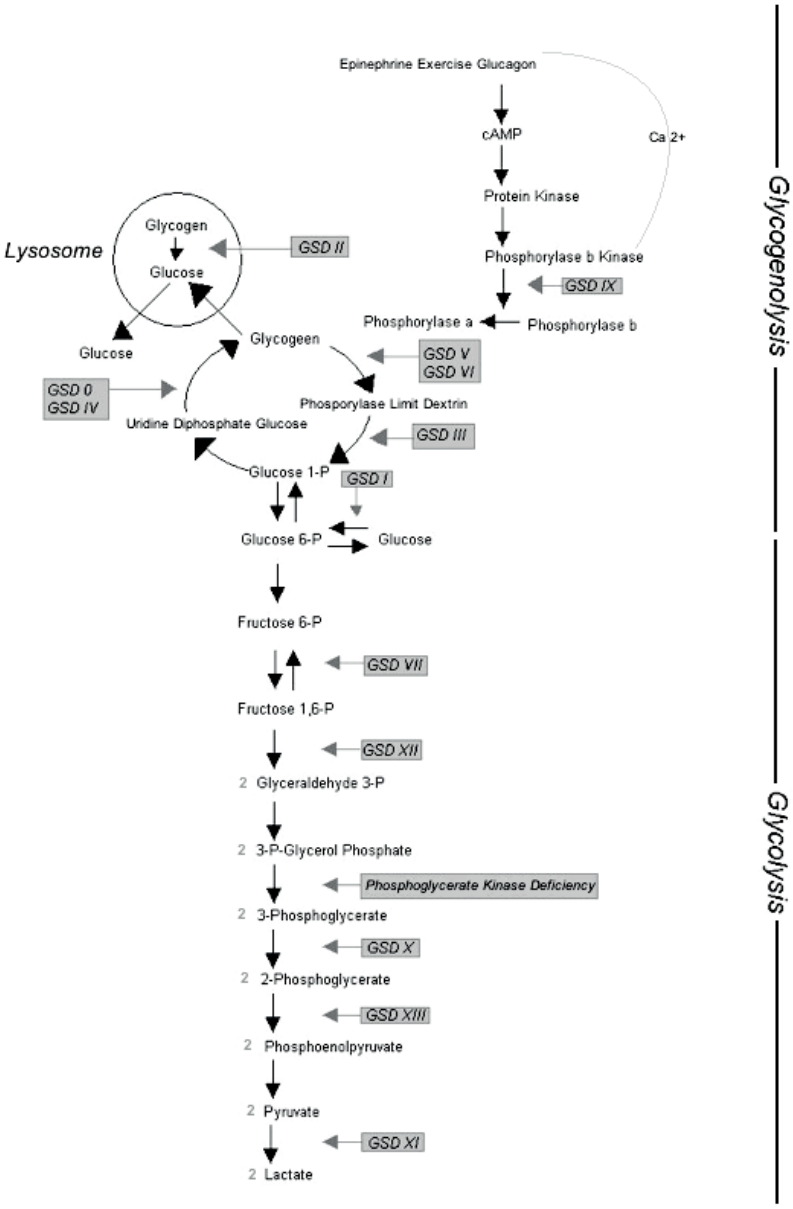
Glycogen storage diseases (GSD) or glycogenoses are a group of inherited metabolic disorders, caused by enzyme defects that regulate glycogen degradation and (paradoxically) glycogen synthesis (figure 1). These disorders primarily involve liver and/or muscle, although rare neurological disorders have been described. GSD are denoted by Roman numeral, or by another synonym which is based on the deficient enzyme or the name of the author of the first description (Laforêt *et al* 2012). The first case of glycogen storage disease type III (GSDIII) was reported in 1928 by Van Creveld who described two patients (Snapper & Van Creveld 1928), and later Van Creveld proved by enzymatic analysis that these patients actually had GSDIII (Van Creveld & Huijing 1964). In 1952, Illingworth and Cori demonstrated excessive amounts of abnormally structured glycogen in liver and muscle tissue of a GSDIII patient (Illingworth & Cori 1952). Analysis of the abnormal glycogen showed that the molecule had short outer chains. Therefore, the glycogen was classified as phosphorylase-limit dextrin, and thus a deficiency of amylo-1,6-glucosidase was predicted – which was confirmed in 1956 (Illingworth *et al* 1956).

The debranching enzyme consists of two active centres that catalyse one of the final steps in the conversion of glycogen to glucose (Bates *et al* 1975). Before the debranching enzyme starts to act, a phosphorylase enzyme breaks four glucose molecules off the glycogen molecule to form limit dextrin (Newgard *et al* 1989). In unaffected cells the first active centre of the debranching enzyme, 1,4-glucan-4-D-glucosyltransferase, transports the outer three glucose molecules of limit dextrin to another chain. Thereafter, the second active centre, amylo-1,6-glucosidase, releases the last glucose molecule (Lui *et al* 1991). In order to establish the diagnosis of GSDIII, mutation analysis can be performed or the debranching enzyme activity can be measured in white blood cells, muscle, and liver tissue. Upon pathologic examination, liver tissue composes of enlarged hepatocytes containing large amounts of limit dextrin. Also, periportal fibrosis and fibrous septa are usually present, particularly in specimens obtained from adult patients.

Four subtypes of GSDIII have been described, the most common form being type IIIa which affects 80% of the GSDIII patients. As GSDIIIa affects both liver, cardiac and skeletal muscle tissue, this subtype is also known as the hepatic-myopatic subtype. The other common subtype is GSDIIIb, affecting 15% of the GSDIII patients. This form of GSDIII is also called the hepatic subtype as in these patients only the liver is affected. Furthermore, there are the two very rare subtypes: GSDIIIc and GSDIIId. In GSDIIIc, there is an active 1,4-glucan-4-D-glucosyltransferase center, but amylo-1,6-glucosidase is deficient. In IIId there is a normal amylo-1,6-glucosidase activity, but 1,4-glucan-4-D-glucosyltransferase is deficient in both liver and muscle tissue. GSDIIId has been described in larger numbers than GSDIIIc, but still both subtypes are considered very rare (Ding *et al* 1990).

GSDIII is an autosomal recessive disorder, in which a mutation in the *AGL* gene (located at 1p21), causes defiency of the debranching enzyme. As reported by Bao *et al* in 1996, the human *AGL* gene is 85 kb in size with 35 exons. In 1997 Bao *et al* recognized the presence of six different isoforms that differ in the 5' end by using several cryptic splice sites upstream of the translation initiation site, which allows the inclusion or removal of exons. Isoform 1 is the generalized form present in the liver, (skeletal and cardiac) muscle, and the kidneys. Isoforms 2, 3, and 4 are only present in skeletal and cardiac muscle tissue. These isoforms are formed as a result of alternative splicing or of a difference in transcription start points. Isoform 1 contains exons 1 and 3; isoforms 2, 3, and 4 start with exon 2. Isoforms 1 through 4 all contain exon 3 which includes the normal initiation codon for protein translation. Exons 4-35 are present in all isoforms (Bao *et al* 1996; Bao *et al* 1997). The glycogen binding site is encoded by exons 31 and 32 and the active site is encoded by exons 6, 13, 14, and 15 (Elpeleg 1999).

Figure 1. Simplified scheme of the glycogenolysis and glycolysis. (Laforêt *et al* 2012). The GSD and their respective enzyme deficiencies are: GSD 0 = glycogen synthase; GSD I = glucose-6-phosphatase; GSD II = α-glucosidase; GSD III = debranching enzyme; GSD IV = branching enzyme; GSD V = myophosphorylase; GSD VI = liver phosphorylase; GSD VII = phosphofructokinase; GSD IX = phosphorylase-b-kinase; GSD X = phosphoglycerate mutase; GSD XI = lactate dehydrogenase; GSD XII = fructose-1,6-biphosphate aldolase A; GSD XIII = β-enolase.



Certain populations have common *AGL* mutations as a result of a founder effect. In GSDIII patients from the Faroe Islands *c.1222C>T* is present (Santer *et al* 2001), and *c.4455delT* in North African Jewish patients (Parvari *et al* 1997). Except for the founder mutations and some other common mutations, most mutations are unique. Missense and splice site mutations, small deletions and insertions, and large intragenic deletions and insertions have been described, many of which produce truncated proteins. The *c.4455delT* mutation in the North African Jewish community generates a truncated protein that is about 97% of its length. This proves that the carboxy terminus, downstream of the glycogen binding site, is essential for normal enzyme function (Parvari *et al* 1997; Dagli *et al* 2010). Individuals with GSDIIIb have mutations in exon 3 of one of their *AGL* alleles. The nonsense mutation *p.Gln6X* and the frameshift deletion *c.17_18delAG* both generate truncated proteins with few amino acids. It is thought that alternative exon or translation initiation in muscle isoforms does not require exon 3, thus leading to normal enzyme activity in the muscles of persons with GSDIIIb who have an exon 3 deletion (Shen *et al* 1996, Elpeleg 1999; Dagli *et al* 2010).

Clinical Findings

In the neonatal period and in infancy the main features of GSDIII are hepatomegaly, hyperketotic-hypoglycaemic episodes after fasting, and hyperlipidaemia. The aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT) levels are elevated during childhood, while the fasting lactate and uric acid levels are normal. Untreated neonates and children are bound to have developmental delay, growth retardation and delayed puberty (Laforêt *et al* 2012). Hepatomegaly is a hallmark feature at the first presentation, but during puberty the size of the liver usually returns to normal, possibly due to progressing fibrosis. Despite this fact, liver complications are still frequent, and the incidence is expected to increase with age (Wolfsdorf and Weinstein 2003). Pathologic examination of liver biopsy samples in adults show signs of (periportal) hepatic fibrosis, and hepatic adenomas are seen in 10-25% of the patients (Labrune *et al* 1997). Severe cases of GSDIII patients who develop liver cirrhosis and related complications such as ascites, oesophageal varices, and even hepatocellular carcinoma have been described as well (Haagsma *et al* 1997; Lee *et al* 1997; Okuda *et al* 1998; Demo *et al* 2007).

GSDIIIa-related myopathy may present in childhood but usually is not clinically evident until patients reach their third decade. GSDIIIa-related myopathy presents as a slowly progressive muscle weakness in the proximal muscles of the shoulder and hip. In some patients there may be clinical muscle weakness of the small distal muscle-groups associated by the development of peripheral neuropathy (Slonim *et al* 1984; Wary *et al* 2010). Cardiomyopathy is a frequent complication in GSDIIIa since limit dextrin can also be stored in myocardial cells and between bundles of myofilaments. This situation causes a form of cardiomyopathy that echocardiographically resembles idiopathic hypertrophic cardiomyopathy, but shows a different response to exercise testing, 24-hour electrocardiographic monitoring and thallium-201 myocardial scintigraphy (Lee *et al* 1997). The clinical significance and long-term consequences of GSDIIIa related cardiomyopathy are unclear due to a lack of data and experience. Recently, the beneficial effects of a high protein diet, in which around 20-30% of total energy is derived from protein, in GSDIIIa-related cardiomyopathy have been described. In three separate case-reports, three severely symptomatic patients with GSDIIIa-related hypertrophic cardiomyopathy improved both clinically objectively on a high protein diet with a limited supply of carbohydrates (Dagli *et al* 2009; Valayannopoulos *et al* 2011; Sentner *et al* 2012).

Treatment

Adequate dietary treatment of GSDIII can improve metabolic control, meaning that hypoglycaemic episodes are prevented, and cholesterol and triglyceride concentrations are decreased (Gremse *et al* 1990; Weinstein and Wolfsdorf 2002). The aim of dietary management in GSDIII patients should be to maintain normoglycaemia by dividing the carbohydrate intake throughout the day. This can be achieved by taking frequent meals and/or regular doses of uncooked cornstarch. Uncooked cornstarch has a compact chemical structure consisting of glucose molecules, which can be slowly degraded, providing a stable glucose supply. Extra protein supplementation is advised as protein may be used in gluconeogenesis in fasting conditions (Slonim *et al* 1984; Wary *et al* 2010). Also, several case-reports have demonstrated that the implementation of a protein-enriched diet has reduced, and even reversed hypertrophic cardiomyopathy in GSDIIIa patients (Dagli *et al* 2009; Sentner *et al* 2011; Valayannopoulos *et al*. 2011). During night-time the use of uncooked cornstarch in combination with protein-enriched feeds can prevent nocturnal hypoglycaemic episodes and improve metabolic control. In GSDIII the intake of products containing galactose and fructose does not have to be restricted, as these short glucose polymers can be processed normally (Weinstein & Wolfsdorf 2002). Severe hypoglycaemic episodes should be treated immediately on admission to the emergency room by intravenous infusion of 10% dextrose with 0,5 normal saline administered at 1,5 times the maintenance rate. Serum concentrations of glucose, electrolytes, and ketones should be monitored. Ketosis should be corrected, as it can induce vomiting and worsen the catabolic state (Dagli *et al* 2010).

Discussion

In GSDIII patients the incidence of hypertrophic cardiomyopathy, skeletal myopathy, and hepatic complications are the main cause of morbidity and mortality (Weinstein & Wolfsdorf 2002). The prognosis of every individual GSDIII patient varies, but in general the patients with GSDIIIb have a better prognosis compared to GSDIIIa patients, probably because in GSDIIIb the muscles are unaffected. Unfortunately, there is little information on the natural clinical course of GSDIII. Over time it became apparent that the development of liver fibrosis, cirrhosis and even hepatocellular carcinoma's have a severe impact on the prognosis, as well as the progressive (cardio)myopathic and neuropathic complications (Slonim *et al* 1984; Ugawa *et al* 1986; Hattori *et al* 1995; Lee *et al* 1997; Kiechl *et al* 1999; Demo *et al* 2007). GSDIII is a very rare condition, with an estimated prevalence in Europe of 1:80,000, and because of this each academic metabolic centre does not have enough patients to investigate the natural course. Therefore, the current state of knowledge is based on single centre experiences in small populations, and most treatment regimens are based on 'best practice'. Even in the light of these findings, life expectancy in GSDIII has improved considerably over the past decades.

Outline of this thesis

The following research questions have been addressed:

- Chapter 2: Is the DGGE method reliable for *AGL* mutation analysis? Are there (novel) genotype-phenotype relationships?
- Chapter 3: What is the natural history of GSDIII, in terms of diagnosis, management, clinical course and outcome?
- Chapter 4: What is the incidence and clinical course of hyperlipidaemia in GSDIII patients?
- Chapter 5: Can cardiac hypertrophy and severe heart failure be reversed in an adult GSD IIIa patient by low-calorie high-protein adjustments?
- Chapter 6: What are differences of muscle ultrasound density and muscle force between patients with GSDI and GSDIII, and how does myopathy progress these patients?

Finally, in chapter 7 a summary along with a discussion and future perspectives are presented.

Chapter 1 is partially adopted from: Dagli A, Sentner CP, Weinstein DA. Glycogen Storage Disease Type III. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Ledbetter N, Mefford HC, Smith RJH, Stephens K, editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2017. 2010 Mar 9 [updated 2016 Dec 29].

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Mutation analysis in glycogen storage disease type III patients in the Netherlands – novel genotype-phenotype relationships and five novel mutations in the *AGL* gene

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JIMD Reports 2013;7:19-26

Summary

Glycogen Storage Disease type III (GSD III) is an autosomal recessive disorder in which a mutation in the *AGL* gene causes deficiency of the glycogen debranching enzyme. In childhood it is characterized by hepatomegaly, keto-hypoglycaemic episodes after short periods of fasting, and hyperlipidaemia. In adulthood myopathy, cardiomyopathy and liver cirrhosis are the main complications. To determine the genotype of the GSD III patients (n=14) diagnosed and treated in our centre, mutation analysis was performed by either denaturing gradient gel electrophoresis or full gene sequencing. We developed, validated and applied both methods, and in all patients a mutation was identified on both alleles. Five novel pathogenic mutations were identified in seven patients, including four missense mutations (*c.643G>A, p.Asp215Asn*; *c.655A>G, p.Asn219Asp*; *c.1027C>T, p.Arg343Trp*; *c.1877A>G, p.His626Arg*), and one frameshift mutation (*c.3911delA, p.Asn1304fs*). The *c.643G>A*, *p.Asp215Asn* mutation is related with type IIIa, as this mutation was found homozygously in two patients. In addition to five novel mutations, we present new genotype-phenotype relationships for *c.2039G>A, p.Trp680X*; *c.753_756delCAGA, p.Asp251fs*; and the intron 32 *c.4260-12A>G* splice site mutation. The *p.Trp680X* mutation was found homozygously in four patients, presenting a mild IIIa phenotype with mild skeletal myopathy, elevated CK values, and no cardiomyopathy. The *p.Asp251fs* mutation was found homozygously in one patient presenting with a severe IIIa phenotype, with skeletal myopathy, and severe symptomatic cardiomyopathy. The *c.4260-12A>G* mutation was found heterozygously, together with the *p.Arg343Trp* mutation in a severe IIIb patient who developed liver cirrhosis and hepatocellular carcinoma, necessitating an orthotopic liver transplantation.

Introduction

Glycogen storage disease type III (GSDIII; OMIM no. 233400) is an autosomal recessive disorder in which mutations in the *AGL* gene cause deficiency of amylo-1,6-glucosidase, 1,4α-D-glucan 4-α-glycosyltransferase, also known as the glycogen debranching enzyme (GDE; EC no. 3.2.1.33 and 2.4.1.25). GDE catalyses the last step in the conversion of glycogen to glucose, and GDE deficiency thus causes storage of an intermediate form of glycogen called limit dextrin (LD) (Smit *et al* 2006). In the IIIa subtype, muscle and liver tissue are deficient in GDE, and this affects 85% of GSDIII patients. Approximately 15% of the patients have type IIIb, in which only the liver is deficient in GDE (Shen *et al* 1996). In the neonatal period and in infancy, the main features are hepatomegaly with elevated aspartate transaminase (ASAT) and alanine transferase (ALAT) values, keto-hypoglycemic episodes after relatively short periods of fasting, and hyperlipidaemia. Untreated neonates and children have developmental delay, growth retardation, and delayed puberty. In puberty and early adulthood, myopathy becomes the predominant feature of GSDIII; the disease presents as a slowly progressive muscle weakness in which the proximal muscles of the shoulder and hip joints are affected. Clinical muscle weakness in the upper and the lower limb muscles can develop in later adulthood, which may be worsened by the development of peripheral neuropathy (Hobson-Webb *et al* 2010; Wolfsdorf & Weinstein 2003). LD can also be stored in heart muscle, which causes a form of cardiomyopathy that resembles idiopathic hypertrophic cardiomyopathy on an echocardiogram (Lee *et al* 1997; Akazawa *et al* 1997).

GDE is composed of 1532 amino acid residues, and has two catalytic centres (Bao *et al* 1996; Lui *et al* 1991). Before GDE starts to act, a phosphorylase enzyme separates four glucose molecules from the glycogen molecule to form LD (Newgard *et al* 1989). Then the first catalytic centre of GDE, 1,4-glucan-4-D-glucosyltransferase, transports the outer three glucose molecules of LD to another chain. The second catalytic centre, amylo-1,6-glucosidase, then releases the last glucose molecule (Ding *et al* 1990). GSDIII can be diagnosed biochemically by measuring GDE activity in skin fibroblasts and/or leucocytes. GDE activity and LD content can also be measured in liver- and/or muscle biopsies (Wolfsdorf and Weinstein 2003). LD content can be measured in erythrocytes.

The *AGL* gene is located on chromosome 1p22, and contains 35 exons covering 85 kb of DNA (Yang-Feng *et al* 1992). The full cDNA is 7 kb with a 4596 bp coding region. A total of six mRNA isoforms are created by alternative splicing. Isoform 1 is the major isoform and widely expressed, including in liver and muscle tissue (Bao *et al* 1996). Isoforms 2, 3, and 4 are present in muscle and cardiac muscle and are formed by alternative splicing or because of the difference in transcription start points. Isoform 1 contains exons 1 and 3. Isoforms 2, 3, and 4 start with exon 2. Isoforms 1 through 4 all contain exon 3, which includes the normal initiation codon for protein translation. Exons 4 through to 35 are present in all isoforms (Bao *et al* 1996; Bao *et al* 1997). The glycogen binding domain is encoded by exons 31-34. The 1,4α-D-glucan 4-α-glycosyltransferase catalytic site is encoded by exons 6, 13, 14, and 15. The amylo-1,6-glucosidase catalytic site is encoded by exons 26 and 27 (Shen *et al* 2002).

Molecular analyses of GSDIII patients have been performed in several ethnic populations and over 100 different *AGL* mutations have been described (Goldstein *et al* 2010), but new mutations are still being reported (http://www.hgmd.org). No clear genotype-phenotype relationship has been established so far, although there is a relation between mutations in exon 3 and the IIIb subtype (Shen *et al* 1996; Shen *et al* 2002). It is unclear, however, what mechanism enables patients with mutations in exon 3 to retain GDE activity in muscle tissue. A possible explanation was proposed by Goldstein *et al* (2010) in which the exon 3 mutation is bypassed using a downstream start codon, thus creating an isoform without the exon 3 mutations.

To determine the genotype of the GSDIII patients diagnosed and treated in our centre (n=14), the University Medical Centre Groningen (UMCG), the Netherlands, we performed mutation analysis by one of two methods. Denaturing gradient gel electrophoresis (DGGE) was applied on eight patients, and full gene sequencing was applied on six patients. We developed, validated, and applied both methods. Here we describe five novel mutations found in seven patients and their phenotypes, and evaluate the phenotype-genotype relationships in patients with previously described mutations.

Materials and Methods

Patients – For this study we analysed 14 patients diagnosed with GSDIII (seven females and seven males). They were diagnosed enzymatically by measuring GDE activity in leukocytes, fibroblasts, and/or liver tissue, and/or muscle tissue. All the patients were diagnosed and are being treated in the UMCG. Their clinical data was collected.

Mutation analysis by DGGE – Genomic DNA was isolated from EDTA blood (Miller *et al* 1988). Primers were designed on the GenBank genomic reference sequence (NW_012865) to include at least 40 bp of intronic sequence on the front end of every exon and at least 20 bp at the back end. Analysis with NGRL Manchester's SNP database (http://ngrl.manchester.ac.uk/SNPCheckV2/snpcheck.htm) confirmed that no known single nucleotide polymorphisms were situated under the primers. The primer specificity was checked and verified in complete genomic sequences with NCBI's ePCR (http://www.ncbi.nlm.nih.gov/projects/e-pcr). We developed fifty primer sets to analyze 30 of the 33 coding exons by DGGE (primer sequences available upon request). The three remaining exons were sequenced directly, as designing DGGE primers with an appropriate melting curve was not possible. Per amplicon the amplification mix consisted of 1.0 µl genomic DNA (40 ng/µl), 10 µl Amplitaq Gold ® Fast PCR Master Mix (Applied Biosystems, California, USA), 3.0 µl primer (3 pmol/µl), and 6.0 µl milliQ water, the reaction volume per sample was 20.0 µl. The samples were amplified by PCR in 96-well plates on a 96 well Gene Amp® 9700 PCR system (Applied Biosystems, California, USA). The PCR program used was: 3 minutes at 96°C, 45 cycles of 1 minute at 96°C, 1 minute annealing at multiple temperatures, 1 minute elongation at 72°C, with a final extension step at 72°C lasting 5 minutes. The annealing temperatures were 5 cycles at 60°C, 5 cycles at 56°C, 5 cycles at 52°C and 30 cycles at 50°C. The PCR products were analyzed by DGGE (Hayes *et al* 1999). Amplicons showing an aberrant banding pattern were sequenced on an ABI 3730 automated sequencer (Applied Biosystems, California, USA) using specific primers. DNA samples from 38 GSD III patients with 34 known mutations were used to validate the DGGE system – and all mutations were detected in our system.

Sequence analysis – We designed 33 primer sets according to the criteria used in conformation sensitive capillary electrophoresis (CSCE, table 1) and applied them for sequencing all coding exons of the gene including flanking intronic sequences. Per sample the amplification mix consisted of 2 µl genomic DNA, 5 µl Amplitaq Gold® Fast PCR Master Mix (Applied Biosystems, California, USA), and 3.0 µl primer (150 fmol/µl). The reaction volume per sample was 10 µl, the samples were amplified by PCR in 384-well plates on a Veriti 384-well thermal cycler (Applied Biosystems, California, USA). The PCR program was: 3 minutes at 94°C, 35 cycles of 1 minute at 94°C, 1 minute annealing at 60°C, 1 minute at 72°C, and a final extension step at 72°C lasting 7 minutes, after which the samples were cooled down to 20°C. 5 µl of the PCR products were loaded with 5 µl loading buffer and run on a 2% agarose gel with a FastRuler Low Range DNA ladder (Fermentas, Vilnius, Lithuania) for comparison. The

remaining PCR products were purified with ExoSAP-IT (Amersham Pharmacia Biotech, Biscataway, NY, USA) and subjected to direct sequencing on an ABI 3730 automated sequencer (Applied Biosystems, California, USA) using specific primers.

Table 1. Sequences of the primer set designed according to the criteria of primer design used in conformation sensitive capillary electrophoresis (CSCE). Primer and amplicon criteria: Optimal primer length 20bp (minimum 18bp; maximum 27bp), optimal annealing temperature 58◦C (minimum 52◦C; maximum 64◦C), optimal GC% 55 (maximum 70), optimal amplicon size 400bp (minimum 200bp; maximum 464bp), maximal amplicon GC% 73. A PT1 tail was added to every forward primer sequence (‘5-‘3; TGTAAAACGACGGCCAGT), and a PT2 tail was added to every reverse primer sequence (‘5-‘3; CAGGAAACAGCTATGACC).

Amplicon	Forward Primer Sequence 5’-3’	Reverse Primer Sequence 5’-3’	Size bp
3	CGAACATGTAAGTGCCGCTGTCA	AGAACACAGCACCATCTTTGCACAA	380
4	GTAGTGCCAAAACAGCATTAGGTTTGC	GCACTGCCATGGTTCATACAGTAACAT	457
5	TTCCATTAAGTTTTGTTGCAAC	CTGCAATGAGAGAATGGACTAATACAC	435
6	TGAACCCAAGTGTTTGACCTCTTTTCC	CCTTTCTCTTATTTGTGTGTATATGTG	432
7	AACTTTTCCTGTAACAGTATCATCG	AATACAGGTTCTAAGTAATTTTCAACC	429
8	GCACTTTGGCGTTTCTCCTGTGA	GACGTTACCCAAAAGAGAGTTTTCCCT	460
9	GGGAGGAGGTAGGAGGATAC	CACATATAGAAACATGGCCCACACACA	456
10	CTGTGTGTGGGCCATGTTTCTATATGT	TTCCCAAAAGGCAATTAACTGCCTGAA	409
11	CTGCATTTCTCCATCTGCTCTAGCAA	ATTTAAGAAATGTACTGAACTCACATG	440
12	CATCCTGCTAGATTTACTCAAAAAGCC	ACCAATAGACTAATGGGGAAGAAAATC	432
13	TTAAAAACCAGTGTTTCCTTGAAG	AATGCTTGTGTCCAACTAGC	381
14	TATGTCAAATCATGCCTCCTTTTGTC	GAAATGAGGTATCTTACCCCAAAGTAG	428
15	CCATTTCTCCAGTTAAGTTATGGG	TGGGTATGATTGTGACCAAGTGTCAGA	445
16	GGTCACAATCATACCCATATACTTC	AAACCACTGAAATCTGGACAAAGG	442
17	CTATGGCATGTTGTGCTAGTGGAAGT	TCCACATACACCTGAGAAGCAGAAAGA	433
18	AGGAGCTTGGAGCCAAGGGTTT	CCATCATACCTGGCCAAGTTACCAAA	447
19	GATTTGAAACCACTTTAGCCTTCC	TGTGGCAACTCCAGCTTGTTTAAC	340
20	TGGGACTCTCATCTTACTACTGTG	GCATGTGGATCAAGACTAACTCTG	340
22	TTGAAAACTTGTCTCCAGGAAGTG	TGGACCGTACTTTGAGTAGCAAGGAT	402
22	GAATGCTGAGTTCCTAAAACATACAC	TGCAACCCAAGTAGGCATACTCTGA	366
23	TTGTGGACTGGGTAGCCCTTGT	GAAGGAAGGAGGAAAATGGTTCAGGTT	397
24	CCTCCTTCCTTCATCATCTTTCAG	CTATCCACCTACAAGCCTTTTCAG	413
25	TGGGTGAAATGAAAGCAGTTTTG	AAAATCTTGAGTAGCATTACAAGC	458
26	ACCCCAGGTTTAGAGTAACTGTTC	CTACCTAAAGAAAATACAGCTCCC	323
27	CAAAAGTGACTGGTTTTTGTCTTC	GGTGCCAAATCAATACTGACATTTG	440
28	CTGGCCTCACCCCAATTCCTATTTC	ATTATATCGTGAGGTTTGGCACAC	352
29	CAAACTGAGCTTTAGAGTGGTTGTCCT	AGATGAAGGGAAGAAGGCAGGGAAAT	398
30	TTCATTACAATTGTTTACCGAATGCCC	GGGTTTTCCGATATTAGCTGATAG	301
31	CACATCTCAATTCAGACTGGCCACAT	AACAAATGGGAATAAGGAACTAAGC	441
32	GGCTTTCCTAACTTCTACGGCCAAAA	AGATGGCATCTCCTTTTGTTGCCC	407
33	TGCCGAGCTTATTCTGTAGAAGAC	AGGCCACAGCCACTCCTAAAAAAG	333
34	TCACCAAGGACCTGTAAGAATTTC	CCTAGGGCATACAGAAATCAATTC	350
35	CACTAGAAGGCAAAAATCACCAGGTCT	AACTTGAGCCTGTGCATATAAGGCATT	294

Analysis of the pathogenicity of novel mutations – The pathogenicity of novel mutations was assessed using six separate methods. 100 control chromosomes from mixed ethnicity were checked for the novel mutations. Conservation of the mutated nucleotide and amino acid was graded with Alamut Version 1.4 (©Interactive Biosoftware). The University of Harvard’s PolyPhen program (http://genetics.bwh.harvard.edu/pph/) predicted the impact of an amino acid substitution on the structure and function of a human protein. The SIFT program (http://blocks.fhcrc.org/sift/SIFT.html) predicted whether an amino acid substitution affects protein function based on sequence homology and the physical properties of amino acids. Finally, we assessed whether the mutation was located in an exon encoding the glycogen binding domain (exon 31-34), or encoding a catalytic site (exon 6, 14 16, 26-27), and measured the GDE activity.

Results

Patients – Of our 14 GSDIII patients, four were related to one other patient (two sisters, and two brothers), all other patients were unrelated. Patient 7 was born to consanguineous parents. Nine patients with type IIIa, two with type IIIb, and three patients were too young to be subtyped based on their clinical presentation. The clinical and biochemical characteristics of the patients are presented in table 2.

Table 2. Demographic, clinical and biochemical characteristics of the analyzed GSD III patients. [#] M = Male, F = Female; [%] OLT = Orthotopic Liver Transplantation

Patient Nr.	Age (yrs)	Sex[#]	Ethnic origin	GDE residual activity (%)	Liver complications	Cardiologic complications	Skeletal muscle complications	Most recent CK value (U/L)
1	8	M	Caribbean	0	Hepatomegaly	None	Proximal myopathy	1083
2	25	F	Caribbean	0	Hepatomegaly	None	None	2232
3	26	M	Caribbean	0	Hepatomegaly	Septal hypertrophy	None	893
4	40	F	Caribbean	0	Hepatomegaly	None	None	3729
5	15	F	Mediterranean	0	Hepatomegaly	None	None	662
6	20	F	Mediterranean	0	Hepatomegaly	None	None	1342
7	32	F	Mediterranean	0	Hepatomegaly	Severe symptomatic left ventricular and septal hypertrophy	Exercise intolerance, distal myopathy	1898
8	3	M	Caucasian	1	Hepatomegaly	None	None	133
9	30	F	Caucasian	0	None	None	Exercise intolerance, distal myopathy	2257
10	30	M	Caucasian	0	Hepatomegaly	None	None	68
11	41	F	Caucasian	0	Hepatomegaly	None	Exercise intolerance	392
12	41	F	Caucasian	0	None, the patient is post-OLT[%], pre-OLT[%] liver cirrhosis and hepatocellular carcinoma was present	None	None	70
13	3	M	Caucasian	0	Hepatomegaly	None	None	128
14	1	M	Caucasian	0	Hepatomegaly	None	None	466

Results of mutation analysis – The patients were fully analyzed and we detected two mutations in each patient (table 3). Five novel mutations were identified in seven patients, including two sisters who were homozygous for *c.643G>A, p.Asp215Asn*, and two brothers who were homozygous for *c.3911delA, p.Asn1304fs*. The other three patients were compound heterozygous with a novel missense mutation and a previously reported pathogenic mutation: *c.655A>G, p.Asn219Asp* in combination with *c.4529dupA, p.Tyr1510X*; *c.1027C>T, p.Arg343Trp* in combination with the splice mutation *c.4260-12A>G*, and *c.1877A>G, p.His626Arg* in combination with *c.1222C>T, p.Arg408X*.

Table 3. Mutation analysis results in fourteen GSD III patients.

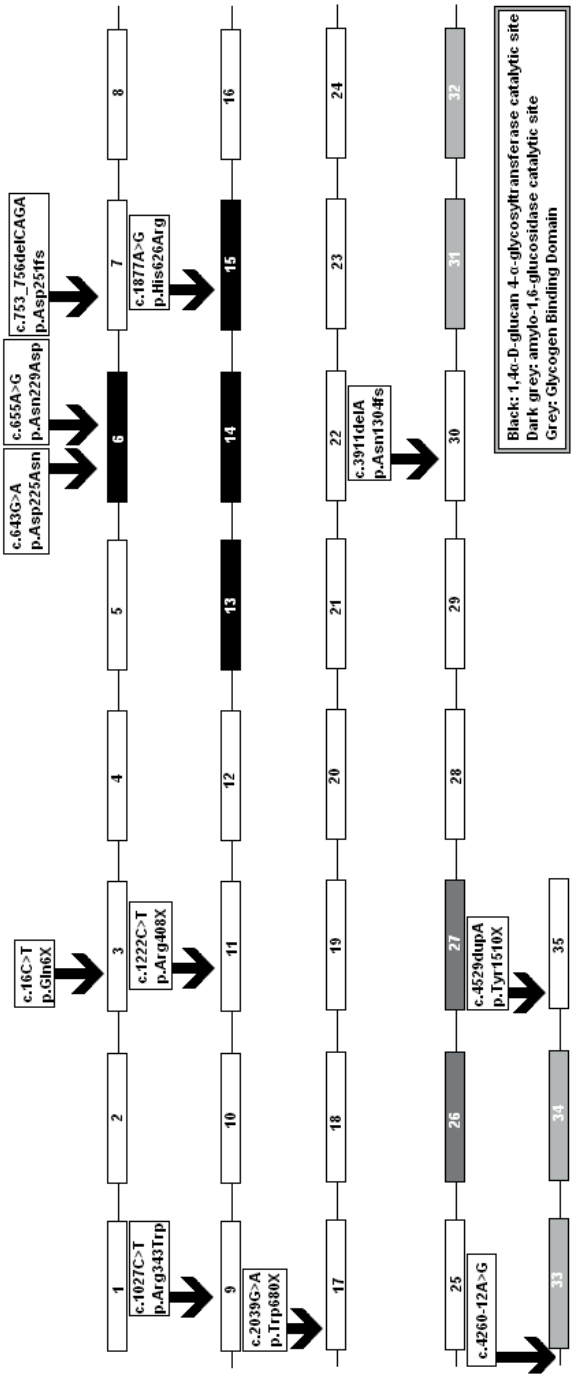
Patient nr.	Exon nr.	Nucleotide change allele 1	Amino acid change allele 1	Mutation type	Exon nr.	Nucleotide change allele 2	Amino acid change allele 2	Mutation type	Mutation Analysis Method
1	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
2	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
3	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	DGGE
4	17	c.2039G>A	p.Trp680X	Nonsense	17	c.2039G>A	p.Trp680X	Nonsense	Sequencing
5	6	c.643G>A	p.Asp215Asn	Missense	6	c.643G>A	p.Asp215Asn	Missense	DGGE
6	6	c.643G>A	p.Asp215Asn	Missense	6	c.643G>A	p.Asp215Asn	Missense	DGGE
7	7	c.753_756delCAGA	p.Asp251fs	Frameshift	7	c.753_756delCAGA	p.Asp251fs	Frameshift	DGGE
8	6	c.655A>G	p.Asn219Asp	Missense	35	c.4529dupA	p.Tyr1510X	Nonsense	Sequencing
9	35	c.4529dupA	p.Tyr1510X	Nonsense	35	c.4529dupA	p.Tyr1510X	Nonsense	DGGE
10	3	c.16C>T	p.Gln6X	Nonsense	3	c.16C>T	p.Gln6X	Nonsense	DGGE
11	11	c.1222C>T	p.Arg408X	Nonsense	15	c.1877A>G	p.His626Arg	Missense	Sequencing
12	9	c.1027C>T	p.Arg343Trp	Missense	Intron 32	c.4260-12A>G		Splice	Sequencing
13	30	c.3911delA	p.Asn1304fs	Frameshift	30	c.3911delA	p.Asn1304fs	Frameshift	Sequencing
14	30	c.3911delA	p.Asn1304fs	Frameshift	30	c.3911delA	p.Asn1304fs	Frameshift	Sequencing

Results of pathogenicity analysis – We consider the four new missense mutations to be pathogenic for several reasons. We did not detect the new mutations in 100 control chromosomes, and these mutations were not found in the NCBI SNP database. Highly conserved amino acids were mutated, and all mutations were predicted to affect protein function by the PolyPhen and SIFT programs. Additionally, three of the four new mutations (*Asp215Asn*; *p.Asn219Asp*; and *p.His626Arg*) were located in an exon encoding the 1,4α-D-glucan 4-α-glycosyltransferase catalytic site of GDE (figure 1). This makes a pathogenic effect of these mutations on GDE function very probable (Cheng *et al* 2009). As the *c.3911delA, p.Asn1304fs* causes a frameshift with the new reading frame ending in a stop codon at position 10, this is considered to be a pathogenic mutation as well. Clinically, all the patients presented with a GSDIII phenotype, and this was biochemically confirmed by enzymatic analysis that no GDE activity was measured to be present.

Table 4. Evidence of the pathogenic effect of the novel missense mutations reported.

Mutation	Conservation of the amino acid	Occurrence in 100 control alleles	Polyphen prediction	SIFT prediction	Location
c.643G>A, p.Asp215Asn	High	Absent	Probably damaging	Not tolerated	Active site, exon 6
c.655A>G, p.Asn219Asp	High	Absent	Probably damaging	Not tolerated	Active site, exon 6
c.1027C>T, p.Arg343Trp	Moderate	Absent	Probably damaging	Not tolerated	Not in an active site, exon 9
c.1877A>G, p.His626Arg	High	Absent	Probably damaging	Not tolerated	Active site, exon 15

Figure 1. The location of all found mutations in the gene, the catalytic sites of GDE are depicted as well; three of the four novel mutations (*Asp215Asn*; *p.Asn219Asp*; and *p.His626Arg*) were located in exons encoding the 1,4α-D-glucan 4-α-glycosyltransferase catalytic site.



Discussion

Here we describe five novel pathogenic mutations in the *AGL* gene, four of which are missense mutations, very likely causing GSDIII in seven patients. Missense mutations causing GSDIII are scarce, as truncating mutations compose the majority of the pathogenic mutations. However, pathogenic missense mutations have been described. Cheng *et al* showed that missense mutations located in the active sites produce a GSDIII phenotype in which there is total or partial abolishment of GDE activity depending on the location of the mutation (Cheng *et al* 2009).

GSDIII is characterized by clinical and genotypical heterogeneity, and clear genotype-phenotype correlations are rare. We therefore present the clinical characteristics of the patients with novel mutations, and discuss the correspondence between their clinical presentation and that described in the literature for the mutations that have been previously described.

Genotype-phenotype analysis – Patients 1 to 4 all had type IIIa and were homozygous for *p.Trp680X*. The phenotype was similar in all four patients, with hepatomegaly, no or mild cardiac involvement, and mild skeletal myopathy with elevated CK values. This suggests a link between the *p.Trp680X* mutation and the type IIIa phenotype. The *p.Trp680X* mutation was described previously in a compound heterozygote patient, in whom it was linked to the IIIb phenotype (Shen *et al* 1996), which is in contrast to what we found in our patients. Interestingly, all our patients with the *c.2039G>A, p.Trp680X* mutation were from the same topographic region and ethnic origin, making a founder effect feasible, as seen for the *c.1222C>T, p.Arg408X* mutation on the Faroe Islands (Santer *et al* 2001). However, in order to prove this, haplotyping for these patients would be required, which we have not done in this study.

Patient 5 (female, age 15 years) and patient 6 (female, age 20 years) were sisters whose parents are from the Mediterranean, who had type IIIa and were homozygous for the novel *p.Asp215Asn* mutation. Their phenotype is mild with hepatomegaly as the main finding, there is no myopathy or cardiomyopathy. In laboratory investigations ASAT, ALAT and CK values were elevated, but there was no hypoglycaemia or hyperlipidaemia. The mild phenotype was probably due to their young age and the good metabolic control of these patients.

Patient 7 (female, age 32 years) had type IIIa and was found to be homozygous for *p.Asp251fs*. She had a severe phenotype, with hepatomegaly, and skeletal muscle involvement with severe exercise intolerance. There was also severe cardiac involvement with hypertrophy of the interventricular septum and left ventricle, necessitating pharmacological treatment and ICD placement. This mutation was previously described in a 3 year-old female, who was found to have hepatosplenomegaly and hypoglycaemia but no myopathy or cardiomyopathy (Lucchiari *et al* 2006).

In patient 8 (male, age 3 years) the novel mutation *p.Asn219Asp* was found to be heterozygous. The other mutation *p.Tyr1510X* was previously reported and associated with a severe IIIa phenotype (Shen *et al* 1997). At first presentation (age 1.5 years), he had severe hepatomegaly extending 15 cm below the costal margin in the medioclavicular line, regular keto-hypoglycaemic episodes and hyperlipidaemia. These symptoms improved dramatically upon starting dietary treatment after diagnosis at the age of 1.5 years with frequent meals during the day and overnight gastric drip feeding. These findings are typical for GSDIII patients in this age group, and the normal CK value does not exclude future muscle involvement, making it hard to assess the subtype or to determine the phenotype that goes with this novel mutation. Furthermore, the latter proves difficult because the patient was found to have compound heterozygous mutations. Patient 9 (female, 30 years) had type IIIa was found to be homozygous

for *p.Tyr1510X* and had a severe GSDIIIa phenotype. She had a severe phenotype including distal myopathy and severe exercise intolerance with elevated CK (2257 U/L). Despite the absence of hepatomegaly, transaminases remained elevated (ASAT 237 U/L, ALAT 175 U/L). This is in concordance with a suggestion made by Shen *et al* that this mutation is associated with a severe GSDIIIa phenotype (Shen *et al* 1997).

Patient 10 (male, 30 years) had type IIIb and was homozygous for *p.Gln6X*. His current phenotype is mild, with hepatomegaly, normal CK values and no cardiac- or skeletal-muscle involvement. This mutation was previously described and is strongly linked with the GSD IIIb phenotype, as are other mutations in exon 3 (Shen *et al* 1996; Shen *et al* 2002). Paradoxically, this patient first presented at the age of 2 years with proximal myopathy and severe hypotonia, but without cardiomyopathy or elevated CK values. His myopathy and hypotonia improved dramatically upon starting dietary treatment, and he has had no muscular symptoms since.

Patient 11 (female, age 41 years) had type IIIa and was compound heterozygous for the novel *p.His626Arg* mutation. Her other mutation *p.Arg408X* was previously described (Santer *et al* 2001; Lam *et al* 2004) and associated with the GSD IIIa phenotype. This corresponds with her phenotype: hepatomegaly and elevated transaminases. She has no proximal or distal myopathy but she does suffer from exercise intolerance and has elevated CK values.

Patient 12 (female, age 41 years) had type IIIb and was compound heterozygous for the novel *p.Arg343Trp* mutation and *c.4260-12A>G* in intron 32. *c.4260-12A>G* was previously described and associated with IIIb as well as IIIa (Okubo *et al* 1998; Shaiu *et al* 2000). Her phenotype was severe IIIb and a case report on her was published after she received an orthotopic liver transplantation after being diagnosed with liver cirrhosis (Haagsma *et al* 1997). Hepatocellular carcinoma was found upon pathological examination of the excised liver. She has never had any muscle involvement, and CK values have always been normal.

Patients 13 (male, age 3 years) and 14 (male, age 1 years) were brothers and homozygous for the novel mutation *c.3911delA*, *p.Asn1304fs* in exon 30. Except for prominent hepatomegaly and the need for appropriate dietary requirements, both patients were generally well. There was no clinical muscle involvement, even though patient 14 had elevated CK-values. As these are pediatric patients it is not yet possible to assess the subtype based on the clinical findings.

Conclusions – We identified two separate mutations in each of our 14 GSDIII patients. Five were novel pathogenic mutations considered to be causal. As we also analyzed parts of the intron sequences (40 bp on the front end, and 20 bp at the back end of every exon), we assume that the chance that we missed other disease-causing mutations is slim. The novel *c.643G>A*, *p.Asp215Asn* mutation is related with type IIIa, as this mutation was found homozygously in two patients both clinically presenting as type IIIa. We also established new genotype-phenotype relationships between *c.2039G>A, p.Trp680X* and type IIIa; *c.753_756delCAGA, p.Asp251fs* and type IIIa; and between the intron 32 *c.4260-12A>G* splice site mutation and type IIIb. The association between *c.4529dupA, p.Tyr1510X* and type IIIa complies with previous literature. However, as the GSD III subtype is not yet clear for every patient, it was not possible to establish a genotype-phenotype relationship for every novel mutation. There is still a large clinical and genotypical heterogeneity in GSD III, which makes establishing genotype-phenotype relationships for GSD III difficult. The fact that we found five new mutations in a relatively small number of GSD III patients further accentuates the need for more genotyping, and indicates that there are probably numerous unidentified mutations.

Acknowledgements
We thank Professor René Santer of the University Clinic Hamburg, Germany for providing the DNA samples for the validation of our methods.

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Chapter 3

Glycogen storage disease type III: diagnosis, genotype, management, clinical course and outcome

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Journal of Inherited Metabolic Disease 2016 Sep;39(5):697-704.

Summary

Glycogen storage disease type III (GSDIII) is a rare disorder of glycogenolysis due to *AGL* gene mutations, causing glycogen debranching enzyme deficiency and storage of limited dextrin. Patients with GSDIIIa show involvement of liver and cardiac/skeletal muscle, whereas GSDIIIb patients display only liver symptoms and signs. The International Study on Glycogen Storage Disease (ISGSDIII) is a descriptive retrospective, international, multi-centre cohort study of diagnosis, genotype, management, clinical course and outcome of 175 patients from 147 families (86 % GSDIIIa; 14 % GSDIIIb), with follow-up into adulthood in 91 patients. In total 58 *AGL* mutations (non-missense mutations were overrepresented and 21 novel mutations were observed) were identified in 76 families. GSDIII patients first presented before the age of 1.5 years, hepatomegaly was the most common presenting clinical sign. Dietary management was very diverse and included frequent meals, uncooked cornstarch and continuous gastric drip feeding. Chronic complications involved the liver (hepatic cirrhosis, adenoma(s), and/or hepatocellular carcinoma in 11 %), heart (cardiac involvement and cardiomyopathy, in 58 % and 15 %, respectively, generally presenting in early childhood), and muscle (pain in 34 %). Type 2 diabetes mellitus was diagnosed in eight out of 91 adult patients (9 %). In adult patients no significant correlation was detected between (non-) missense *AGL* genotypes and hepatic, cardiac or muscular complications. This study demonstrates heterogeneity in a large cohort of ageing GSDIII patients. An international GSD patient registry is warranted to prospectively define the clinical course, heterogeneity and the effect of different dietary interventions in patients with GSDIII.

Introduction

Glycogen storage disease type III (GSDIII; OMIM #232400) is a rare inborn error of glycogen degradation with an incidence of 1:100,000 (Dagli *et al* 2010; Kishnani *et al* 2010; Laforêt *et al* 2012). GSDIII is caused by mutations in the *AGL* gene and the subsequent deficiency of the glycogen debranching enzyme (GDE; EC no. 3.2.1.33 and 2.4.1.25, UniProt P35573). GDE contains two catalytic centres that catalyse one of the last steps in the conversion of glycogen to glucose-1-phosphate.

Patients with GSDIII present clinically with hepatomegaly, failure to thrive and fasting intolerance, biochemically associated with ketotic hypoglycaemia. Phenotypically, patients can be further classified into having either GSDIIIa (±85 %), with involvement of the liver, heart and skeletal muscle, or GSDIIIb (±15 %), in which only the liver is affected (Shen *et al* 1996; Laforêt *et al* 2012). Dietary management aims to maintain normoglycaemia and prevent hyperketonaemia by dividing sufficient carbohydrate intake throughout the day, and using additional protein as a substrate for gluconeogenesis (as recently reviewed by Derks & Smit 2015). During long-term follow-up the clinical focus shifts to the prevention and management of progressive hepatic, cardiac and myopathic complications (Dagli *et al* 2009; Sentner *et al* 2012; Verbeek *et al* 2015).

Current knowledge on the clinical course and outcome has been based on case reports and small single centre cohort studies, of mainly young patients, on which the current management guidelines are based (Dagli *et al* 2010; Kishnani *et al* 2010). The International Study on GSDIII (ISGSDIII) is a descriptive, retrospective, international, multi-centre cohort study on the diagnosis, genotype, management, clinical course and outcome in 175 patients with GSDIII, with follow-up into adulthood in 91 patients.

Methods

The Medical Ethical Committee of the University Medical Centre Groningen, the Netherlands approved the study protocol (ref.no. METc2008.035). Patients were included from 17 metabolic centres in ten countries. Between 2007 and 2011 data on GSDIII patients were collected using a case record form (CRF) for every patient and anonymously archived in a database. The CRF was based on the European Study on GSDI (ESGSDI; Rake *et al* 2002), and modified for ISGSDIII by two authors (GPAS, CPS) (for the complete CRF, see Supplemental data 1). The CRFs were filled out either by the treating physician or by one single investigator (CPS).

GSDIII patients were included when an enzyme assay and/or *AGL* molecular analysis had confirmed the diagnosis. GSDIIIa was defined as (a) deficient GDE activity in muscle or (b) clinical and/or biochemical signs of cardiac and/or skeletal muscular involvement. Based on the family history, individual patients could be categorized as proband, symptomatic sibling or neonatally screened patient due to an affected older sibling. To study the relationship between *AGL* genotypes and GSDIII phenotypes, statistical analyses were only performed in adult patients. Cardiac involvement was defined as the presence of abnormalities corresponding to cardiac hypertrophy in the electrocardiographic and/or echocardiographic investigations. Cardiomyopathy was defined as the presence of cardiac hypertrophy in combination with 1) (severe) exercise intolerance (and/or 2) the use of pharmacological treatment for (symptoms of) heart failure.

AGL mutations were grouped according to the type of mutation, i.e. missense or non-missense *AGL* genotypes. Non-missense mutations resulting in either frameshift or splicing modifications were assumed to be pathogenic. Pathogenicity of novel missense mutations was predicted by five methods: Alamut Version 2.2 (©Interactive Biosoftware), PolyPhen-2

(http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.bii.a-star.edu.sg/), whether the mutation was located in the catalytic site (exon 6, 13-15, 26-27) or an exon encoding the glycogen binding domain (exons 31-34), and the NHLBI Exome Sequencing Project (ESP) Exome Variant Server (http://evs.gs.washington.edu/EVS/).

Data were processed with IBM® SPSS® Statistics Version 20 (SPSS Inc., Chicago, Il, USA). The results were expressed as median (range) for non-parametric data, and mean (standard deviation) for parametric data. Differences between normally distributed continuous data were analysed using the unpaired two-tailed T-test. Not normally distributed data were analysed using the Mann-Whitney-U or Kruskal-Wallis test. For dichotomous data, the Fisher's exact test was used. The level of significance was set at $p < 0.05$.

Results

Two hundred and twenty-eight patients with GSDIII were identified. Data from 53 patients were incomplete and not included in the analysis. Table 1 presents demographic and diagnostic information on 175 patients with GSDIII from 147 families, of whom 91 (52 %) had reached adulthood.

Table 1 Distribution of observations between GSDIIIa and GSDIIIb patients.

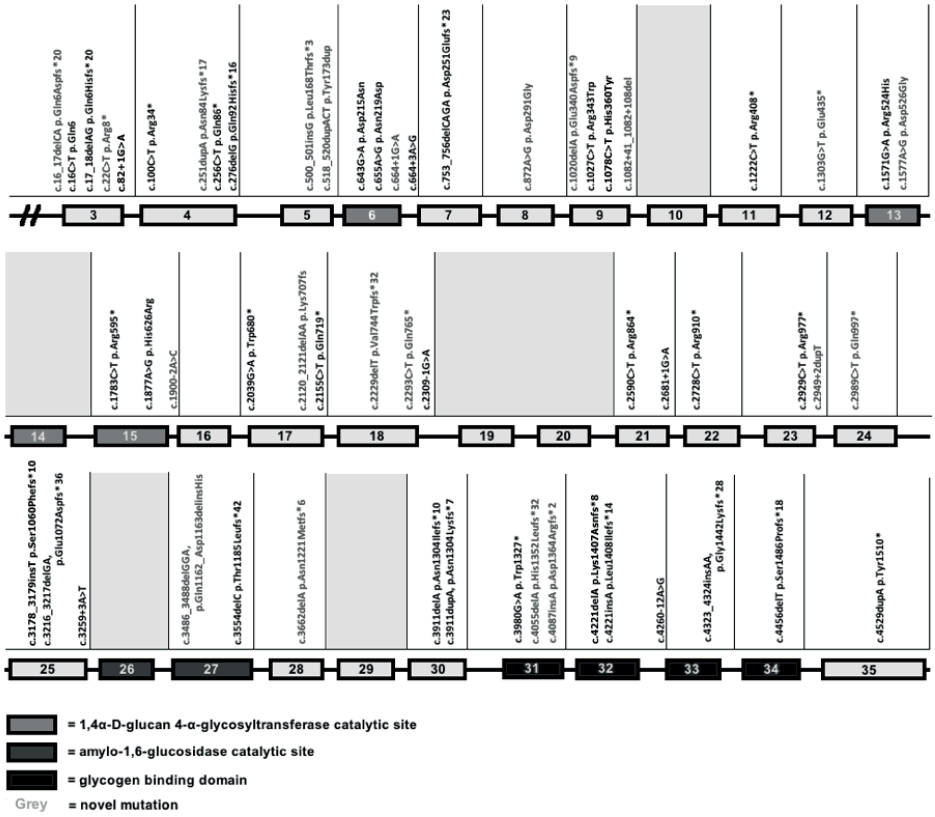
	GSDIIIa	GSDIIIb	Total
Demographic information			
Male/Female (n (%))	69 (46%) / 82 (54%)	13 (52%) / 11 (48%)	82 (47%) / 93 (53%)
Age (yrs: median (range))	20.6 (1–64.1)	16.4 (0.3–50.7)	19.3 (0.3–64.2)
Follow-up (yrs: median (range))	17.4 (0.5–61.1)	14.1(0.2 – 48.7)	16.2 (0.2–61.2)
Ethnicity (n)			
Asian	2	1	3
Caucasian-Mediterranean	125	23	148
African-Caribbean	7	0	7
Mixed	17	0	17

Clinical course and presentation until establishment of the diagnosis

Pregnancy, birth, and presenting symptoms – Post-natal hypoglycaemia was documented for six term, normal birth-weight patients (3 %). In the 147 probands, the median age at the first clinical presentation in GSDIIIa and GSDIIIb was at 0.7 year (range: day 1–8.1 years) and 1.0 year (range: day 1–6.0 years), respectively. Common presenting symptoms included hepatomegaly (98 %), hypoglycaemia (53 %), failure to thrive (49 %) and recurrent illness and/or infections (17 %).

Mutation analysis – Figure 1 presents the 58 individual reported mutations, including 21 novel mutations, depicted per exon/intron. *AGL* gene mutation analysis was performed in 76 out of 147 families, two causative mutations were identified in 72 families (95 %), in four families one allelic mutation was identified. The majority (50/58; 86 %) were non-missense mutations resulting in a truncated protein. Among the probands in whom both affected alleles were identified, 47 were found to be homozygotes, and 23 were compound heterozygotes. No statistically significant correlation was recognized between a (non-) missense *AGL* genotype and the occurrence of major complications (hepatic, cardiac, myopathic) in 49 adult (≥18 years) patients.

Figure 1 *AGL* mutations in the ISGSDIII-cohort depicted per exon/intron.



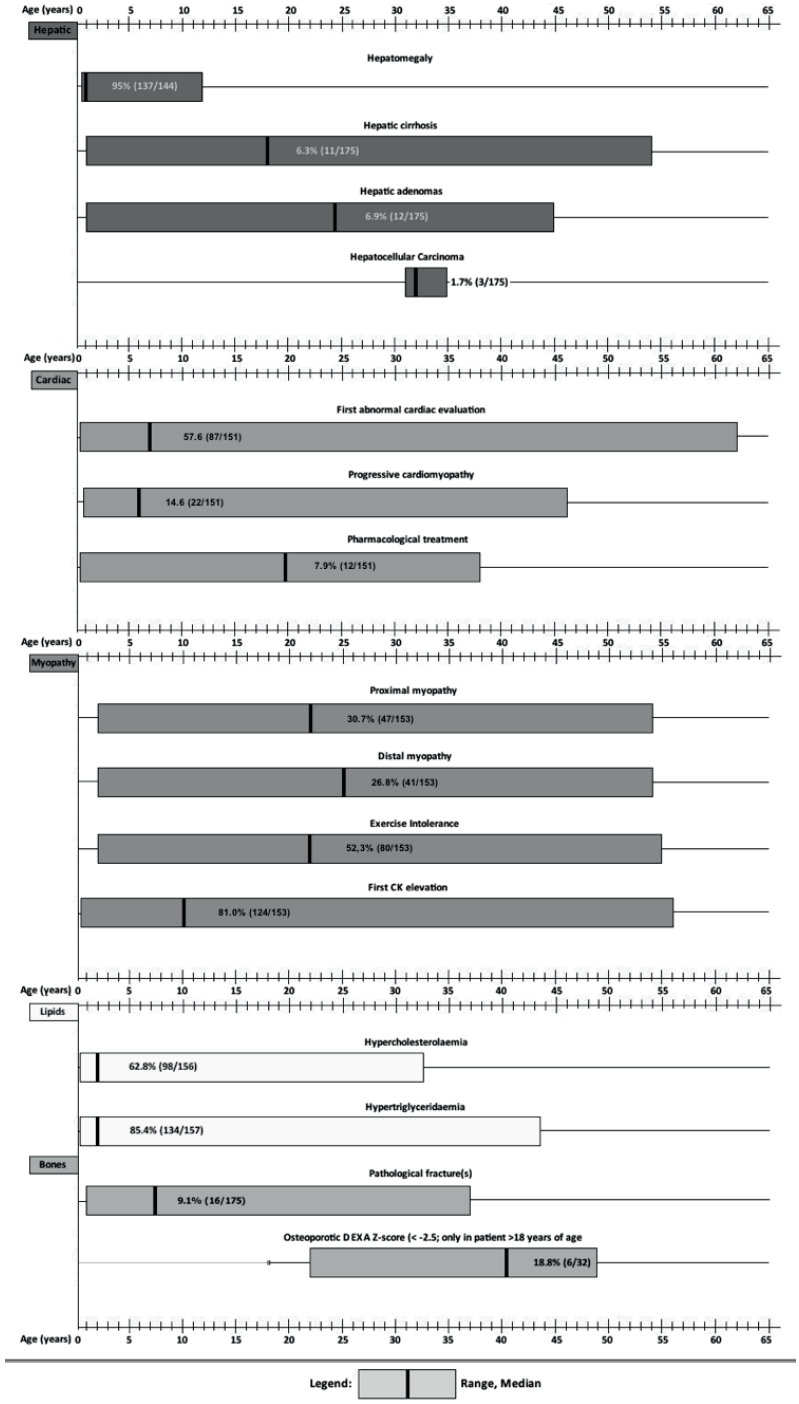
Clinical course after establishment of the diagnosis

Figure 2 presents the age (median and range) of onset of clinical features and complications.

Hepatic complications – The overall prevalence of severe hepatic complications (hepatic cirrhosis, adenomas and/or HCC) was 11 % (19 out of 175 patients; one GSDIIIb; 12 females). In 11 patients hepatic adenomas had been diagnosed (seven females), two of these patients were overweight at the time of diagnosis, and none of these patients used oral contraceptives. Alpha-fetoprotein levels were measured for eight patients, in none of these patients levels were over 40 ng/ml. In four patients with hepatic cirrhosis, orthotopic liver transplantation was performed at a median age of 32 years (15–35; three females; one GSDIIIb). In two of these patients (one GSDIIIb) hepatocellular carcinoma (HCC) was diagnosed postoperatively upon pathological examination. Three patients in total had been diagnosed with HCC, all of whom had progressed from hepatic fibrosis, to cirrhosis, and then HCC.

Cardiac complications – Cardiac involvement was reported in 58 % (87/151) of the GSDIIIa patients. Electrocardiographic and/or echocardiographic signs of left ventricular hypertrophy were found in 61 GSDIIIa patients. The remaining 26 patients had other forms of cardiac hypertrophy (isolated septal, right ventricular or biventricular hypertrophy). Cardiomyopathy was reported in 15 % (22/151) of all GSDIIIa patients. There were no laboratory parameters

Figure 2 Age range of onset of disease features of patients.



(cholesterol, triglycerides, ASAT, ALAT, CK) significantly increased in GSDIIIa patients with or without cardiomyopathy. Heart transplantations had not been performed in this cohort.

Neuromuscular complications – Muscular pain after minimal exercise was reported by 59 patients (34 %). Three patients were wheelchair dependent due to skeletal muscle involvement (signs of permanent muscle weakness with atrophy). Electromyography (EMG) data were available from 33 patients and mainly showed myopathic changes, such as fibrillations and positive sharp waves. In 124 GSDIIIa patients (81 %) CK concentrations were elevated at some point at a median age of 10 years (0.3 – 56.1). Cardiomyopathy was associated with a significantly higher prevalence of distal myopathy (Fisher's exact $p = 0.034$) and muscular pain after minimal exercise (Fisher's exact $p = 0.030$).

Growth and development – Adult height was reached at a median age of 19 years (15–23) (Table 2). There was no significant difference between GSDIIIa and GSDIIIb patients ($p = 0.18$). Of the patients who had reached adult height 24 % (21/86) had a BMI over 25 kg/m2 at the latest follow-up, i.e. overweight according to WHO criteria. Pubertal development was delayed in 36 patients, and there was no significant difference between the subtypes ($p = 0.72$). Furthermore, no significant difference was found in adult height in SDS between the patients who had delayed compared to normal pubertal development ($p = 0.15$).

Table 2 Height in SDS (compared to age-, gender- and ethnically corrected reference values) at latest follow-up in all patients.

Age group (yrs)	N	Median Height SDS (range)	N (%) < -2.0 SDS
0.0 – 2.0	10	-0.16 (-2.45 – 0.60)	1 (10)
2.0 – 5.0	20	-1.19 (-2.96 – 1.56)	2 (10)
5.0 – 10.0	27	-0.71 (-2.86 – 1.65)	5 (19)
10.0 – 15.0	24	-0.56 (-1.99 – 1.06)	-
15.0 – 20.0	26	0.26 (-1.81 – 2.50)	-
> 20.0	61	0.13 (-2.86 – 2.46)	2 (3)
Total	168	-0.31 (-2.96 – 2.50)	10 (6)

Bones – Bone fractures were reported in 16 patients with median age 7.5 years (range 1-18). According to WHO criteria (T-scores), 14 GSDIIIa patients had normal bone mineral density (BMD), 12 patients had osteopenia and six patients had osteoporosis (all GSDIIIa patients). Significantly increased CK (Kruskal-Wallis $p = 0.004$) and ALAT (Kruskal-Wallis $p = 0.027$) values were observed in the osteoporosis group when compared with the normal BMD-group.
Hyperlipidaemia – Serum cholesterol and triglyceride concentrations were measured at the latest follow–up for 154 and 156 GSDIII patients, respectively. Cholesterol concentrations were consistently elevated (>5.17 mmol/L) in 34 % of the evaluated patients across different age groups. Triglyceride concentrations were mainly elevated (>1.69 mmol/L) in early childhood (in 23/29 79 %) patients up to 5 years. Between the age of 5 and 15 years this incidence decreased to 72 % (31/43), and after the age of 15 years stabilized to 40 % (34/84). Complications due to hyperlipidaemia, such as atherosclerosis or pancreatitis, were not reported in this population.

Endocrinologic complications – Type 2 diabetes mellitus (DM2) was diagnosed in eight out of 91 adult patients (9 %; all GSDIIIa), at median age of 38 years (32–44). Three patients were treated with insulin, and five with dietary adjustments. Four of these patients were overweight (BMI >25 kg/m^2). HCC was not reported in patients with type 2 diabetes mellitus. Hirsutism

was described in one patient. Irregular menses or amenorrhea was described in 19 patients. Polycystic ovaries were diagnosed by ultrasound in five patients at a median age of 19 years (6–30).

Mental and social development – Mental development was reported as normal in the majority of the patients, six patients displayed low or borderline intelligence (3 %) and one patient displayed severe developmental delay. Of the patients over 15 years of age (n = 101), 50 patients (50 %) had normal employment and 23 patients (23 %) were following normal general education. Sixteen patients (16 %) were unemployed, with eight of these patients being unemployed due to mental and/or physical disability. Thirty patients (21 females) had 64 healthy children.

Mortality – Three patients with GSDIIIa had died because of cardiomyopathy: one patient because of severe congestive heart failure at the age of 1 year; and two patients because of sudden cardiac death due to progressive cardiac fibrosis at the age of 29 and 39 years, respectively (Ramachandran *et al* 2012). One patient with GSDIIIa died at the age of 36 years because of severe liver failure due to end-stage hepatic cirrhosis in combination with hepatocellular carcinoma. One patient died of causes unrelated to his GSD.

Dietary treatment

Dietary treatment at latest follow-up – Information regarding dietary treatment was collected on 171 patients (98 %; 87 children). Dietary restrictions were imposed on 16 patients; lactose restriction in ten patients and fat intake restriction in ten patients; 96 patients were reported to have a protein-enriched diet. In children (n = 49) the median cumulative protein intake was 2.9 grams per kilogram bodyweight per day (gr/kg/d; range 0.5–4.4), and in adults (n = 47) the median cumulative protein intake was 1.7 gr/kg/d (range 0.9–3.0). Impaired dietary compliance was reported by the treating physician and/or dietician in at least 29 patients (17 patients aged < 25 years).

History of dietary treatment – One hundred forty-six patients (83 %) were treated immediately after diagnosis. One hundred twenty-two patients were treated with uncooked cornstarch (UCCS) at some point; treatment with UCCS was stopped in 27 patients. Ninety-five patients were treated with UCCS at the latest dietary adjustment (64 patients age <18 years). Fifty-seven patients were treated with continuous gastric drip feeding (CGDF) at some point; CGDF was stopped in 18 patients. Thirty-nine patients were treated with CGDF at the latest dietary adjustment (33 patients age <18 years). In total 17 patients had received no dietary treatment at all (nine patients age <25 years).

Discussion

ISGSDIII is a descriptive, retrospective, international, multi-centre cohort study of 175 patients. This study addresses important issues regarding clinical presentation and follow-up into adulthood.

Before discussing the results, some methodological issues need to be addressed. First, as ISGSDIII is a retrospective study, we have predominantly collected cross-sectional rather than longitudinal patient data, and there has been missing data. Unfortunately, IGSDIII did not collect data on fasting tolerance. Second, the ISGSDIII cohort is still relatively young, and follow-up has not extended into adulthood for all patients. This may have caused an

underrepresentation of long-term complications. Third, as most participating centres and colleagues are centre of expertise, selection bias towards relatively severely affected patients may have affected the results. Fourth, patients with the extremely rare subtypes GSDIIIc (presumably the result of glucosidase debranching deficiency) and GSDIIId (presumably the result of transferase debranching deficiency) have not been included. Last, despite the use of a CRF, in different centres clinical and laboratory data are not yet recorded in a standardized and quantitative manner (for instance dietary parameters, echocardiographic parameters, quantification of skeletal muscle strength and exercise tolerance). Particularly the availability of dietary management data has been very limited. It additionally needs to be recognized that there may be a difference between prescribed diets and daily practice.

In contrast to the previous reports on GSDIII patients (Dagli *et al* 2010; Kishnani *et al* 2010; Laforêt *et al* 2012), ISGSDIII demonstrates that hypoglycaemia is a presenting symptom in just half of the patients. Therefore, in patients with a traditional clinical (hepatomegaly) and biochemical (elevated transaminase values, hyperlipidaemia) presentation, the diagnosis of GSDIII should not be rejected in the absence of (severe) hypoglycaemia. In addition, the finding of ketotic hypoglycemia after short fasting test is a major argument for GSDIII, as demonstrated recently (Hoogeveen *et al* 2015). Severe mental retardation and mortality due to metabolic derangement are uncommon in GSDIII patients. ESGSDI has reported high morbidity and mortality because of metabolic derangements with hypoglycaemia (Rake *et al* 2002). The difference between these studies may be partially explained by the relatively younger cohort of ISGSDIII. More importantly, there is a fundamental difference in metabolic compensation between GSDI patients (alternative lactate accumulates quickly, in the absence of ketones) and GSDIII patients (gluconeogenesis is intact and ketones can gradually be formed) during fasting.

Clear genotype-phenotype correlations are rare in GSDIII. The association between exon 3 mutations and GSDIIIb has been reported previously (Shen *et al* 1996; Elpeleg 1999; Shen & Chen 2002; Goldstein *et al* 2010). Interestingly, non-missense *AGL* mutations are overrepresented in the ISGSDIII cohort, whereas in most metabolic diseases missense mutations predominate. It can be hypothesized that missense mutations in the large *AGL* gene cause only minor reduction of GDE enzyme activity. Moreover, ISGSDIII demonstrates that GSDIIIa patients display a more severe clinical course than GSDIIIb patients. The latter group clinically presents at a later stage and has fewer complications, such as hepatic cirrhosis and HCC. The large ISGSDIII cohort did not identify additional correlations between *AGL* genotype and severe complications.

In accordance with previous reports (Vertilus *et al* 2010) ISGSDIII demonstrates that cardiac hypertrophy is common in GSDIIIa patients, mostly starting in the first decade of life (Fig. 2). Cardiac involvement remains stable over time in the majority of the affected patients, with even a portion of the patients regressing to normal values (data not presented). Hence, functional and clinically relevant hypertrophic cardiomyopathy is rare in GSDIIIa patients. Observations from GSDIIIa patients with severe hypertrophic cardiomyopathy suggest an important role of macronutrient intake (Derks & Smit 2015). To date it is speculative which macronutrient intervention is dominant, because each of the following has been described, i.e. decreased total caloric intake (Sentner *et al* 2012), increased protein intake (Dagli *et al* 2009; Sentner *et al* 2012), increased fat intake (Brambilla *et al* 2014), ketone bodies (Valayannopoulos *et al* 2011) and Atkins diet (Mayorandan *et al* 2014). It is not possible to draw causative conclusions from these single case observations, because increasing one macronutrient (either protein or fat) without affecting the other, inevitably affects the remaining macronutrient (carbohydrates). Based on at least two arguments it can be hypothesized that carbohydrate overtreatment may be an important risk factor for cardiac involvement and/or

cardiomyopathy. First, decreased carbohydrate intake was the intervention shared by the above-mentioned reports in which cardiomyopathy resolved after dietary intervention. Secondly, decompensated structural cardiomyopathy is most frequently reported around the time of highest endogenous glucose requirements (i.e. childhood) and the prescription of relatively high amounts of dietary carbohydrate.

ISGSDIII demonstrates that GSDIII patients have (severe) growth retardation in (early) childhood, but eventually reach normal adult height. There is no significant difference in growth between GSDIIIa and GSDIIIb patients, suggesting that the metabolic demands on gluconeogenesis in GSDIII in general are more important than the presence of a muscular GDE deficiency.

ISGSDIII demonstrates a high incidence of bone fractures in paediatric GSDIII patients, suggesting the development of reduced BMD at an early age. The pathophysiology of reduced BMD is unclear, but an association with specific nutritional deficiencies in GSDIII (Folk & Greene 1984; Kishnani *et al* 1999), and reduced metabolic control in GSDI (Rake *et al* 2003) are mentioned. Recently, ALAT has been suggested to be a marker for metabolic control in GSDIII (Dagli *et al* 2010). ISGSDIII demonstrates a negative correlation between osteopenia/osteoporosis and ALAT, supporting the hypothesis that metabolic control affects BMD.

ISGSDIII reports a higher incidence of DM2 in ageing GSDIII patients than in the general population (i.e. 9 %: compared to 6 % in the general population in the western world according to the World Diabetes Foundation). Previous case reports and case studies have described the association between DM2 and GSDIII (Moe *et al* 1972; Oki *et al* 2000; Ismail 2009; Sharma 2009; Spengos *et al* 2009) but the aetiology is largely unknown. In the ISGSDIII cohort, half of the diabetic patients are obese, suggesting that decreased insulin sensitivity might play a role. Second, the constant intake of carbohydrate enriched nutrients to maintain euglycaemia may induce insulin resistance.

Conclusions

ISGSDIII presents large heterogeneity between individual GSDIII patients. Most GSDIII patients present clinically in their first year of life with hepatomegaly as the major presenting symptom. From an acute disease in childhood, GSDIII develops into a chronic, progressive disease in adulthood, affecting liver, heart, skeletal muscle and bones. Chronic complications and the risk of developing DM2 emphasize the need of closely following the ageing GSDIII cohort. Standardized quantitative clinical data collection is warranted by an international longitudinal GSD patient registry and biobank.

Acknowledgements
Acknowledgements to the contributors to ISGSDIII: **Germany** Prof K Ullrich, *Hamburg*; **Czech Republic** Prof J Zéman, *Prague*; **Netherlands** Prof D-J Reijngoud, *Groningen*; Dr E Morava, *Nijmegen*; Dr E Rubio, *Maastricht*; Dr G Visser, *Utrecht*; Dr A Bosch, *Amsterdam*; **United Kingdom** Dr M Champion, Dr S Grünewald, Dr L Abulhoul, Dr P Gissen, Dr R Lachmann, Dr Y Rahman, Dr H Mundy *London*; Dr P Newsome, *Birmingham*; A. Dalton, *Sheffield*; **Italy** Prof GP Comi, *Milan*; Dr M Di Rocco, *Genova*; **Canada** Prof C Polychronakos, Dr AM Sbrocchi, *Montreal*

The ISGSDIII collaborative researchers received financial support from Junior Scientific Masterclass (Graduate School GUIDE, University of Groningen, the Netherlands), Metakids (the Netherlands), the National Institutes of Health (NIH), National Center for Research Resources (NCRR; CTSA grant UL1 TR000064; University of Florida), the Johnny Damon Foundation, and the Hope and Fund for GSDIII Research managed through the University of Florida Office of Development.

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Supplement to Chapter 3

ISGSDIII Questionnaire

Questionnaire Nr. ☐☐☐

International Study on Glycogen Storage Disease Type III

ISGSDIII

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International Study on Glycogen Storage Disease Type III Questionnaire

0.1	Investigator			
0.2	Date of completing this form	⌊⌊⌋⌊⌋ ⌊⌊⌋⌊⌋ ⌊⌊⌋⌊⌋ (ddmmyy)		

1.1	Initials patient			
1.2	Date of birth	(ddmmyy)		
1.3	Informed Consent	O no	O yes	

1.4	Siblings with GSD III	O no	O yes	
	If yes: initials and day of birth			

2.1	Hospital	
2.2	Country	
2.3	Patients doctor	

3.1	Sex	O male	O female	

4.1	O alive	O deceased			
	4.2	If alive			
		Present treatment in your hospital		O yes	O no
		If no: name hospital of present treatment :			
		If yes : other hospital involved in treatment			O no
			O yes: name: address:		
	4.3	If deceased	Date of death ☐☐☐☐☐☐ (ddmmyy)		
		Cause of death : Based on clinical date : Pathological findings:			

5 - Pregnancy and birth

5.1	Complications in pregnancy	O no	O yes :
5.2	Complications at birth	O no	O yes :
5.3	Gestational age	☐☐ Weeks	
5.4	Birth weight	☐☐☐☐ Grams	

6 - First symptoms

6.1	First symptom			
	Hypoglycemia		O no	O yes
	Hepatomegaly		O no	O yes
	Developmental delay		O no	O yes
	Biochemical abnormalities		O no	O yes, details:
	Other		O no	O yes, details:
	6.2	At the age of	☐☐☐ (ddmmyy)	
	6.3	At the age of	☐☐☐ (ddmmyy)	

7 - Diagnosis

7.1	Clinical diagnosis			**O** GSD III with myopathy		
				O GSD III without myopathy		
7.2	Based on Histology			Liver biopsy	**O** yes	**O** no
		If yes	Date	☐☐ ☐☐ (mmyy)		
			Name centre			
				Muscle biopsy	**O** yes	**O** no
		If yes	Date	☐☐ ☐☐ (mmyy)		
			Name centre			
7.3	Based on Enzymatic Diagnosis					
	Amilo-1.6 glucosidase deficiency in white blood cells			**O** yes	**O** no	**O** not done
	Amilo-1.6 glucosidase deficiency in liver			**O** yes	**O** no	**O** not done
	Amilo-1.6 glucosidase deficiency in muscle			**O** yes	**O** no	**O** not done
	State subtype of GSD III (if tested)					
	Elevated RBC's Glycogen			**O** yes	**O** no	**O** not done
7.4	Mutation analysis		State mutation if known :			**O** not done

8 - Dietary history & farmacological treatment

8.1	Dietary history until the age of 25 years	Point out in **figure A**	
8.2	If age > 25 years: alterations in dietary treatment after the age of 25	O no	O yes
	If yes : alteration :	At the age of :	
		☐☐ (years)	
		☐☐ (years)	
		☐☐ (years)	
8.3	Comments on dietary history :		
8.4	Farmacological treatment until the age of 25 years	Point out in **figure A**	
8.5	If age > 25 years: alterations in farmacological treatment after the age of 25	O no	O yes
	If yes : alteration :	At the age of :	
		☐☐ (years)	
		☐☐ (years)	
		☐☐ (years)	
8.6	Comments on farmacological treatment:		

9 - Growth

9.1	Nationality		**:**		
9.2	Ethnic group		O Asian	O Caucasian	O Mediterrenean
			O Negroid	O other, nl :	
9.3	Parental height		Father: (cm) ☐☐	Mother: (cm) ☐☐	
9.4	Growth until the age of 23 years	9.4.1	Height (cm)		Fill in **figure B** (physical – examination)
		9.4.2	Height (SD's) *		
		9.4.3	Weight (kg)		
9.5	Adult height		☐☐☐ (cm), reached at ☐☐ (age in years)		
9.6	Pubertal development		O not relevant, too young		O unknown
			O early **	O normal	O late ***
	If female, menarche at the age of		☐☐ (years)		O unknown
9.7	Bone-age measurement		O no	O yes	
	If yes, mention chronological and bone-ages:				

* SD's according to the standards of the country of origin.

** Before 8 years in girls or 9 years in boys.

*** After 14 years in girls or 15 years in boys.

10 - Hypoglycemia

10.1	Admissions because of convulsions and/or coma due to documented hypoglycemia in each period							
	Age	Number of	age	Number of	age	Number of	age	Number of
	0-2 y		2-5 y		5-10 y		10 + y	

11 - Hyperlipidemia

11.1	Blood cholesterol	Fill in **figure C** (laboratory investigations blood)	
11.2	Blood triglycerides		
11.3		O no	O yes, at the age of ☐☐ (years)
	If yes, course :		
11.4	Atherosclerosis	O no	O yes, at the age of ☐☐ (years)
			O yes , at autopsy
	If yes, course, symptoms and findings :		

12 - Hepatic complications

12.1	Cirrhosis	O no	O yes, at the age of ☐☐ (years)	
	Course :			
12.2	Hepatic adenomas	O no	O yes, first detected at the age of ☐☐ (years)	
			O single one	O multiple
	Course :			

13 - Osteopenia

13.1	Pathological fractures	O no	O yes, at the age of ☐☐ (years)
	If yes, course:		

14 - Psychosocial

14.1	Mental development	**O** low {IQ < 65}	**O** borderline {IQ 65-85}	**O** normal {IQ 85-115}	**O** high {IQ>115}
	Current school or most recent finished school :				
14.2	If working – age	**O** unemployed ☐			
		O employed, profession :			
14.3	Married or long – standing relationship	**O** not relevant	**O** no	**O** yes	
14.4	Children		**O** not relevant	**O** no	**O** yes, number ☐

15 - Cardiomyopathy

15.1	Abnormal Electrocardiogram	O not assessed	O no	O yes at the age of ☐☐ (years)
15.2	Abnormal Echocardiogram	O not assessed	O no	O yes at the age of ☐☐ (years)
	Details:			
	Progression		O no	O yes
15.3	Clinical signs of heart failure	O no	O yes at the age of ☐☐ (years)	
15.4	Requirement of medical therapy	O no	O yes, started at the age of ☐☐ (years)	
15.5	Heart transplantation	O no	O yes at the age of ☐☐ (years)	
15.6	Coronary heart disease	O no	O yes at the age of ☐☐ (years)	
15.7	Angina pectoris	O no	O yes at the age of ☐☐ (years)	
15.8	Myocardial infarction	O no	O yes at the age of ☐☐ (years)	

16 - Neuromuscular

16.1	Hypotonia in family	O no	O yes	
16.2	Delayed walking *	O no	O yes	
16.3	Proximal myopathy, muscular wasting	O no	O yes, at the age of ☐☐)	
16.4	Muscle pain	O no	O yes	
16.5	Distal myopathy	O no	O yes, at the age of ☐☐)	
16.6	Exercise Intolerance	O no	O yes	
		Details:		
16.7	EMG (Electromyogram)	O not assessed	O normal	O abnormal at the age of ☐☐
		Details:		
16.8	NCV (Nerve Conduction Velocity)	O not assessed	O normal	O abnormal, details :

* after 18 months of age.

17 - Endocrinology

17.1	Diabetes mellitus / Insulin resistance	O no	O yes, if yes type :
		Age of onset ☐☐ (years)	
		State initiated therapy:	
17.2	Hirsutism	O no	O yes
17.3	Irregular menses/Amenorrhea	O no	O yes
17.4	Polycystic ovary by U.S.	O no	O yes, at age of ☐☐

18 - Renal Function

18.1	Renal Function	O not assessed	O normal	O abnormal, details:
		Age of onset ☐☐		
18.2	Tubular Dysfunction	O no		O yes, details:
18.3	Glomerular Dysfunction	O no		O yes, details:
18.4	Renal Tubular Acidosis	O no		O yes, state type:
		Age of onset ☐☐		
18.5	Other Kidney abnormalities	O no		O yes, details:

Figure A Dietary history & farmacological treatment

Point out the dietary history (till the age of 25 years) in this figure.
Abbreviations used:

FM	frequent meals
CS	cornstarch added to the meals
GDF	gastric drip feeding
TCS	total cornstarch supplementation in grams per kilogram of bodyweight
TPS	total protein supplementation in grams per kilogram of bodyweight
CS1	cornstarch once a night (f.e. before going to sleep)
CS2	cornstarch two times a night (f.e. before going to sleep and at 3.00 a.m.)
H.P.	high protein diet
F.T.	farmacological treatment

Age (years)		**0.5**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Day	FM													
	CS													
	GDF													
	TCS													
	TPS													
Nights	FM													
	CS1													
	CS2													
	GDF													
	TCS													
	TPS													
	H.P.													
	F.T.													

Age (years)		**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**
Day	FM													
	CS													
	GDF													
	TCS													
	TPS													
Nights	FM													
	CS1													
	CS2													
	GDF													
	TCS													
	TPS													
	H.P.													
	F.T.													

Figure B Physical Examination

Fill in date of examination, height in cm, height in standard deviation score (SDS, if known), weight in kg and liver in cm below the costal margin in the midclavicular line.

***** SD's according to the standards of the country of origin.

Age (years)	± 0.6	± 1.0	± 2.0	± 3.0	± 4.0	± 5.0	± 6.0	± 7
Date (ddmmyy)								
Height (cm)								
Height (SD's)*								
Weight (kg)								
Liver in m.c.l b.c.m								

Age (years)	± 8.0	± 9.0	± 10.0	± 11.0	± 12.0	± 13.0	± 14.0	± 15.0
Date (ddmmyy)								
Height (cm)								
Height (SD's)*								
Weight (kg)								
Liver in m.c.l b.c.m								

Age (years)	± 16.0	± 17.0	± 18.0	± 19.0	± 20.0	± 21.0	± 22.0	± 23.0
Date (ddmmyy)								
Height (cm)								
Height (SD's)*								
Weight (kg)								
Liver in m.c.l b.c.m								

Figure C Laboratory investigations blood

Fill in date of investigation, blood cholesterol, triglycerides, uric acid, haemoglobin, and hematocrit, mean corpuscular volume (MCV), calcium, creatinin, cholinesterase, alpha-fetoprotein, troponin, AST, ALT, creatine kinase, CK-MB, CK-MM and state which units are used in each investigation (e.g. mmo1/1).

Age (years)	*units*	*± 0.6*	*± 1.0*	*± 2.0*	*± 4.0*	*± 6.0*	*± 8.0*	*± 10.0*	*± 12.0*
Date (ddmmyy)									
Cholesterol									
Triglycerides									
Uric acid									
Haemoglobin									
Hematocrit									
MCV									
Calcium									
Creatinin									
Cholinesterase									
Alpha-fetoprotein									
Troponin									
AST									
ALT									
Creatine Kinase (CK)									
CK-MB									
CK-MM									

Age (years)	*± 14.0*	*± 16.0*	*± 18.0*	*± 20.0*	*± 22.0*	*± 25.0*	*± .....*	*± ....*	*± ....*
Date (ddmmyy)									
Cholesterol									
Triglycerides									
Uric acid									
Haemoglobin									
Hematocrit									
MCV									
Calcium									
Creatinin									
Cholinesterase									
Alpha-fetoprotein									
Troponin									
AST									
ALT									
Creatine Kinase (CK)									
CK-MB									
CK-MM									

Chapter 4

Hyperlipidemia in glycogen storage disease type III: effect of age and metabolic control

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Journal of Inherited Metabolic Disease 2008 Dec;31(6):729-32.

Summary

While the presence of hyperlipidaemia in glycogen storage disease (GSD) type Ia and Ib is generally accepted, few investigators have adequately assessed lipid profiles of GSD III in children, in whom the presence of hyperlipidaemia may be most prominent. We analysed the lipid profiles in 44 GSDIII patients from 6 months to 30 years of age. Hypertriglyceridaemia and hypercholesterolaemia were common in children younger than 3 years of age. Hypertriglyceridaemia correlated negatively with age, and may reflect increased severity of hypoglycaemia in this younger population. The presence of hyperlipidaemia during childhood in these patients identifies another GSD population that could be at risk for early cardiovascular disease (CVD). Consequently, the outcome of clinical trials investigating the vascular effect of hyperlipidaemia in GSD applies to types other than GSDI.

Introduction

Type III glycogen storage disease (GSDIII; OMIM 232400) results from a defect in glycogen debranching enzyme (GDE) that interrupts glycogenolysis. In the first step of glycogenolysis, glycogen phosphorylase and GDE remove glucose from the outer branches of glycogen to form glucose 1-phosphate. Impairment of GDE leads to the accumulation of an abnormal form of glycogen, limit dextrin, in liver, heart and skeletal muscle. As the biochemical abnormalities in GSDIII are present in these affected organs, creatine kinase (CK), aspartate transaminase (ASAT) and alanine transaminase (ALAT) are used as markers of control in the management of this disorder (Wolfsdorf and Weinstein 2003).

In the United States, 80% of patients with GSDIII have liver, heart and skeletal muscle involvement (type IIIa), while most of the remaining patients have only liver involvement (type IIIb). The predominant manifestation of GSDIII in infancy and childhood is hypoglycaemia with fasting. As patients age, however, muscle damage occurs (type IIIa), and most patients have severe physical limitations by the fourth decade of life (Wolfsdorf and Weinstein 2003).

Unlike the other hepatic GSDs, researchers debate the presence of hyperlipidaemia in GSD III. Geberhiwot *et al* (2007) described the lipid profiles in GSD I, III and IX. He found evidence of pro-atherogenic lipid profiles in GSDIa characterized by hypercholesterolaemia, hypertriglyceridaemia and reduced HDL. However, he found no significant abnormalities in the lipid profiles of a cohort of six patients with GSDIII aged 14–to 54 years. Likewise, Hershkovitz *et al* (1999) reported no abnormality of plasma lipid and lipoprotein profile in 11 GSDIII patients aged 17–54 years. Despite this evidence, the small number of patients studied does not yet justify the conclusion that GSDIII patients have no risk for hyperlipidaemia.

Given that the majority of studies reviewing hyperlipidaemia in GSDIII do not include patients younger than 14 years of age, in whom hypoglycaemia is most severe, we analysed the largest collection of GSDIII patient data available. We ascertained the prevalence of hyperlipidaemia in GSDIII at all ages. We postulated that hyperlipidaemia correlates with hypoglycaemia, which is most severe in early childhood.

Methods

Design – The study was based on a secondary analysis of patient data recorded through the collaborative International Study on GSDIII (ISGSDIII) database. This database was designed to collect outcome data of natural history and clinical interventions to develop clinical guidelines and therapeutic strategies for GSDIII. Clinical and laboratory data from participants are entered annually into this longitudinal database. We included results for total cholesterol, triglycerides and ALAT. These data were analysed within five age groups that were defined before the analysis: 0–3, 3–6, 6–12, 12–18, and greater than 18 years.

Participants – The diagnosis of GSDIII was established either by histological examination of liver biopsy specimens or by enzymatic methods and, more recently, through mutation analysis of DNA obtained from a blood or saliva sample. IRB approval was obtained for the collection of data in the ISGSDIII database, and informed consent with assent of minors was obtained from all participants.

A total of 44 patients (32 GSDIIIa and 12 GSDIIIb) identified from hospital records of 16 metabolic centres in eight countries had a complete set of variables with 204 observations. A quality review check by looking at date of birth and initials ensured that no participant was duplicated. Data from patient records were compiled by either the treating physician or one of the investigators. In order to assess changes with time, at least two observation times were

needed for inclusion of a patient in the analysis. There were 32 individuals with multiple time points for triglycerides and 30 with multiple time points for cholesterol.

Statistical methods – The key inferences were based on personal linear regression slopes to see whether the quantitative outcomes (serum triglycerides and serum cholesterol) were changing over time. Since these may be outlier-prone, we used the Wilcoxon signed-rank test, which is stable against such outliers, to compare with a null value of zero slope in the target population. Secondarily, we correlated the fitted slopes for triglycerides with ALAT to see whether the changes tended to be concordant (positive association) versus discordant (negative association) by the Spearman rank correlation test. All reported p-values are two-sided.

For descriptive purposes, we also provide means and standard deviations for outcomes by age period. To avoid bias, each subject was weighted equally, regardless of the number of observations in the reporting period. The participant's individual mean for the age period was used as the outcome. This method was also applied to estimate the prevalence of hypertriglyceridaemia and hypercholesterolaemia, which are 0=no or 1=yes at each participant's observation point in the age group.

Results

Triglycerides – Prevalence of hypertriglyceridaemia (defined as >1.69 mmol/L) was estimated to be 67% (standard error=6%; Table 1). Elevated triglyceride concentrations, however, were most commonly found in younger children (Fig. 1), with a significant decrease in triglycerides with increasing age (p=0.026). Approximately 75% of the subjects had a negative slope and the median slope was −0.11 mmol/L per year.

Table 1 Prevalence of hypercholesterolaemia and hypertriglyceridaemia by age. This table demonstrates the prevalence of both hypercholesterolaemia and hypertriglyceridaemia within pre-determined age brackets. The prevalence across all age groups for hypercholesterolaemia was 31% and for hypertriglyceridaemia was 67%.

Age in years	Number of patients Chol/TG	Prevalence of hypercholesterolaemia (SE)	Prevalence of hypertriglyceridaemia (SE)
0.0 – 3.0	19/19	58% (10%)	89% (6%)
3.0 – 6.0	15/14	33% (12%)	86% (8%)
6.0 – 12.0	18/17	51% (11%)	87% (7%)
12.0 – 18.0	15/16	22% (9%)	63% (12%)
> 18.0	16/16	22% (9%)	44% (11%)

Cholesterol – Prevalence of hypercholesterolaemia (defined as total cholesterol >5.17 mmol/L) was estimated to be 31% (standard error=6%; Table 1). Unlike triglycerides, however, the elevated cholesterol concentrations did not demonstrate an age predilection (Fig. 2), nor did absolute cholesterol concentrations significantly associate with age (p=0.59) with a median slope of −0.02 mmol/L per year.

Markers of control – Spearman correlation analysis of the individual slopes with age revealed no significant association between changes in ALAT and changes in triglycerides (r=−0.05, p=0.85).

Figure 1 Triglyceride box-and-whisker plot. Boxes represent the 25th to 75th percentiles, and the median is represented in each box. The range of the observations is depicted by the whiskers. The dotted line represents the cut-off for hypertriglyceridemia defined as >1.69 mmol/L (150 mg/dl). One observation – the mean of multiple values – per patient was plotted within each age bracket. N=the number of observations within each age bracket.

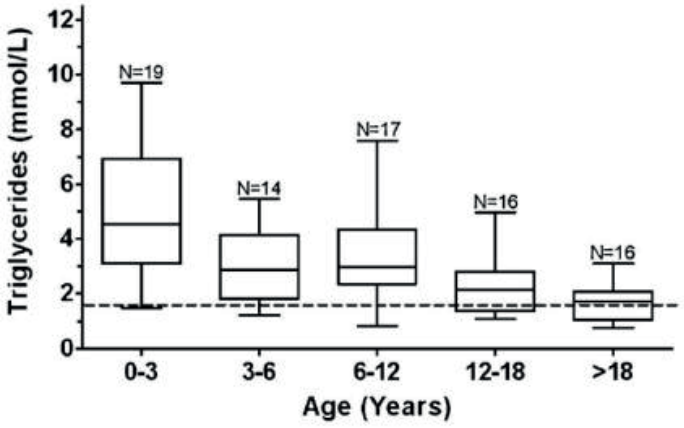
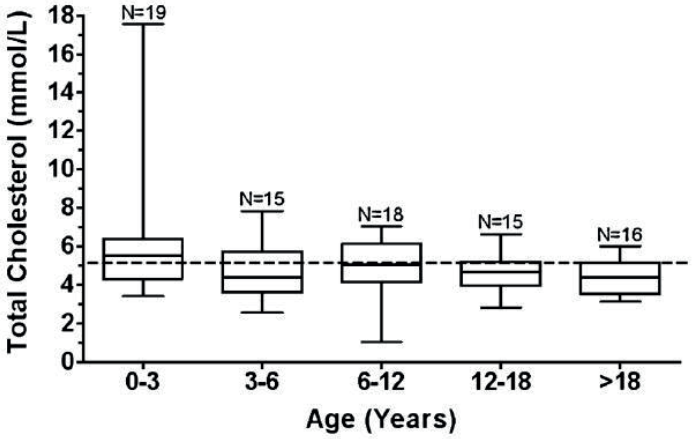


Figure 2 Total cholesterol box-and-whisker plot. Boxes represent the 25th to 75th percentiles, and the median is represented in each box. The range of the observations is depicted by the whiskers. The dotted line represents the cut-off for hypercholesterolemia defined as >5.17 mmol/L (200 mg/dl). One observation – the mean of multiple values - per patient was plotted within each age bracket. N=the number of observations within each age bracket.



Discussion

Hyperlipidaemia in the hepatic GSDs develops from a combination of two mechanisms. The first, specific to GSDI, results from shunting of glycogen breakdown products through the glycolytic pathway, which increases formation of acetyl-CoA and synthesis of fatty acids and cholesterol in the liver (Bandsma *et al* 2008). The second mechanism, which occurs in GSD VI and IX, results from increased β-oxidation of fats in the setting of hypoglycaemia that, in turn, increases fatty acid flux from adipose tissue to the liver as an alternative source of fuel. This second mechanism is hypothesized to result in the observed hyperlipidaemia in GSDIII.

Although the circumstances responsible for hyperlipidemia in other types of GSD are present in GSDIII, hypertriglyceridaemia has not previously been characterized in children with this form of GSD. The inability to mobilize glycogen results in frequent hypoglycaemia in affected children. This frequency of hypoglycaemia in GSDIII patients, however, decreases with age, which may explain why previous investigations did not reveal lipid abnormalities in this population.

With improving dietary management, patients with GSD are living well into adulthood. Patients with GSDI and III commonly reach ages where the potential contribution of hyperlipidaemia to the development of atherosclerosis becomes important. However, even though GSDI patients manifest a pro-atherogenic lipid profile characterized by hypercholesterolaemia, hypertriglyceridaemia, and reduced HDL, investigations have not demonstrated an increased frequency of cardiovascular disease (CVD) in this population (Ubels *et al* 2002). On the basis of early studies (Muhlhausen *et al* 2005; Nguyen *et al* 2006; Wittenstein *et al* 2002) that failed to establish a correlation between GSD patients with hyperlipidaemia and risk of early CVD, the current consensus guideline for management of GSDI does not mandate treatment with lipid-lowering agents unless patients are at risk for pancreatitis. Researchers have attempted to explain the apparent protective features of GSDI by proposing mechanisms such as increased cholesterol efflux, increased antioxidant potential, and decreased platelet aggregation (Muhlhausen *et al* 2005; Nguyen *et al* 2006; Wittenstein *et al* 2002). However, these hypotheses have been developed on the basis of very few patients.

Unlike in patients with GSDI, hypertriglyceridaemia in GSDIII does not reach levels high enough to cause pancreatitis. Indeed, pancreatitis has not been described in GSDIII, and treatment for elevated lipids may be less likely to be commenced. Nevertheless, the potential risk of elevated triglycerides has yet to be determined, especially regarding risk for vessel dysfunction and CVD. If vascular dysfunction does occur, aggressive treatment is warranted. Future studies in the GSDIII population are therefore needed to determine whether the elevated lipids in GSDIII are pro-atherogenic and, if so, whether pharmacological therapy will decrease the risk of CVD.

Limitations of this study arise from the lack of information regarding dietary therapy in this population. In addition, only total cholesterol and triglycerides were included in the database, while HDL and LDL were omitted, resulting in incomplete assessment of cardiovascular risk. Finally, while this study represents the largest assessment of hyperlipidaemia in GSDIII, the numbers are still relatively small, and an assessment of lipids in the subtypes of GSDIII could not be performed. Despite these limitations, hyperlipidaemia clearly occurs in this population, and a more thorough evaluation of cardiovascular risk factors is needed as part of new prospective studies assessing CVD.

In conclusion, hyperlipidaemia occurs in children with GSDIII. The duration of this metabolic derangement throughout childhood may place adolescent and adult patients at risk for CVD. Investigations to assess this risk are warranted.

Acknowledgments

The authors acknowledge and thank the following physicians for contributing to the International Study on GSD III (ISGSDIII) database: E. B. Haagsma (Universitair Medisch Centrum, Groningen), J. Zeman (Charles University, Prague), P. Lee (University College London) and M. Berry (Montreal Children's Hospital, Quebec). Support for this project was provided by the Association for Glycogen Storage Disease, University of Florida Glycogen Storage Disease Research Fund, the National Center for Research Resources General Clinical Research Center grant M01 RR 00082, National Institutes of Health, and NIH Mentored Career Award K23 RR 017560 (D.A.W.).

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Chapter 5

Heart failure due to severe hypertrophic cardiomyopathy reversed by low calorie, high protein dietary adjustments in a glycogen storage disease type IIIa patient

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JIMD Reports 2012;5:13-6.

Summary

In glycogen storage disease type III (GSDIII) deficiency of the debranching enzyme causes storage of an intermediate glycogen molecule (limit dextrin) in the affected tissues. In subtype IIIa hepatic tissue, skeletal- and cardiac muscle tissue is affected, while in subtype IIIb only hepatic tissue is affected. Cardiac storage of limit dextrin causes a form of cardiomyopathy which resembles primary hypertrophic cardiomyopathy on cardiac ultrasound. We present a 32 year-old GSDIIIa patient with severe left ventricular hypertrophy (LVH) first diagnosed at the age of 8 years. LVH remained stable and symptomless until the patient presented at age 25 years with increasing dyspnoea, fatigue, obesity, and NYHA (New York Heart Association) functional classification 2 out of 4. Dyspnoea, fatigue, and obesity progressed, and at age 28 years she was severely symptomatic with NYHA classification 3+ out of 4. On echocardiogram and electrocardiogram the LVH had progressed as well. Initially she was rejected for cardiac transplantation because of severe obesity. Therefore, a 900 calorie, high protein diet providing 37% of total energy, was prescribed during four months on which 10 kilograms weight loss was achieved. However, her symptoms as well as the electrocardiographic and echocardiographic LVH indices had improved dramatically – ultimately deferring cardiac transplantation. Thereafter the caloric intake was increased to 1370 calories per day, and the high protein intake was continued providing 43% of total energy. After three years of follow-up the patient remains satisfied with reasonable exercise tolerance and minor symptoms in daily life.

Introduction

Glycogen storage disease type III (GSDIII) is an autosomal recessive disorder in which a mutation in the *AGL*-gene causes deficiency of the glycogen debranching enzyme (GDE). The DE consists of two active centres, which catalyze the last step in the conversion of glycogen to glucose (Smit *et al* 2006). The absence of DE activity in GSD III patients causes storage of an intermediate form of glycogen, limit dextrin (LD) (Chen 2001). 85% of the GSDIII patients have subtype IIIa in which GDE is deficient in muscle and liver tissue. 15% of the patients have subtype IIIb in which GDE is deficient in the liver (Shen *et al* 1996). In neonates and infants the main features are hepatomegaly, keto-hypoglycemic episodes after short periods of fasting, and hyperlipidemia. Poorly treated neonates and children have developmental delay, growth retardation and delayed puberty. Proximal and distal myopathy presents in adult GSDIIIa patients, which may be enforced by the development of peripheral neuropathy (Wolfsdorf and Weinstein 2003). Cardiomyopathy is a frequent complication in GSDIIIa since LD can also store in myocardial cells and between bundles of myofilaments (Moses *et al* 1989; Smit *et al* 1990; Labrune *et al* 1991; Coleman *et al* 1992; Carvalho *et al* 1993; Talente *et al* 1994). This causes a form of cardiomyopathy that echocardiographically resembles primary hypertrophic cardiomyopathy due to sarcomere gene mutations, but shows a different response to exercise testing, 24-hour electrocardiographic monitoring and thallium-201 myocardial scintigraphy (Lee *et al* 1997; Akazawa *et al* 1997; Olson *et al* 1984). The clinical significance and long-term consequences of GSDIIIa related cardiomyopathy are unclear due to a lack of data and experience.

The aim of the dietary treatment of GSDIII is to divide the carbohydrate intake throughout the day to maintain normoglycemia by taking frequent meals and regular cornstarch doses (Gremse *et al* 1990). Protein supplementation is necessary as it serves as a substrate for gluconeogenesis during fasting conditions and improves myopathy and growth failure (Slonim *et al* 1982; Slonim *et al* 1984; Kiechl *et al* 1999). However, there is no consensus between centres on the usage of a high protein diet or the amount of cornstarch that should be provided.

In this case-report we present a GSDIIIa patient with severely symptomatic hypertrophic cardiomyopathy which was reversed after initiating a low-calorie, high protein diet to achieve weight loss for a cardiac transplantation preparation program. Subsequently her cardiomyopathy-related symptoms and signs improved dramatically and cardiac transplantation could be deferred.

Case Report

The patient reported is a 32 year-old Turkish female born to consanguineous parents after an uncomplicated pregnancy and birth. She was diagnosed with glycogen storage disease in Turkey at a young age, but subtyping for GSDIII was only initiated at the age of 8 years after she moved to the Netherlands. Enzymatic measurements confirmed absent DE activity in muscle- and liver tissue, confirming GSDIIIa. Genetic mutation analysis revealed a pathologic homozygote 4 basepair deletion in exon 7 of the *AGL* gene (GeneBank genomic reference sequence NW_012865) c.753_756delCAGA causing a frameshift (Lucchiari et al 2006). Upon clinical evaluation the main findings were hepatomegaly with elevated aspartate transaminase (203 U/L), alanine transferase (253 U/L), triglycerides (1,7 mmol/L) and creatin kinase values (2112 U/L). A grade III out of VI systolic cardiac murmur was heard in the 4th left intercostal space. Further cardiac evaluation revealed hypertrophic cardiomyopathy with concentric left ventricular hypertrophy (LVH). On the ECG no rhythm- or conduction disturbances were seen. Following diagnosis, dietary treatment was initiated with protein-enriched frequent meals during the day and one late night meal. She responded well to treatment and no hypoglycaemic

episodes requiring hospitalisation have occurred since. During puberty the liver size normalized, but the transaminase values remained elevated. Echocardiographically the LVH remained stable and symptomless, therefore no further cardiologic follow-up was deemed necessary.

At age 25 years the patient presented in the outpatient-ward with increasing dyspnoea, fatigue, obesity, and functional classification according to the New York Heart Association (NYHA) 2 out of 4 (table 1). At physical examination, her heart rate was 69 bpm, blood pressure 100/60 mmHg, with mild jugular venous pressure elevation. Her electrocardiogram showed increased QRS-voltage and -duration with negative T-waves comparable with severe LVH (figure 1). The Sokolow-Lyon-, Cornell Voltage- and Romhilt-Estes electrocardiographic indices for LVH were all positive (figure 1). The echocardiogram showed severe concentric LVH with intraventricular septum (IVS) thickness of 22 mm, and a posterior wall (PW) thickness of 18 mm with normal dimensions (table 1). She was treated with low-dose furosemide, fluid restriction, and her diet was adjusted to provide extra protein during the day. Also, a late night feed combined with cornstarch was added to ensure normoglycemia during the night. In the following years the symptoms of dyspnoea and fatigue slowly progressed, and she gained weight. At age 28 years, she was severely symptomatic with a NYHA functional class of 3+ out of 4. The patients' BMI increased to 32.2 because she avoided physical exercise as this provoked palpitations and chest pain. The electrocardiographic and echocardiographic LVH indices worsened accordingly (IVS thickness 32mm, PW thickness 25mm). At that time lowdose perindopril and carvedilol to the furosemide, and a prophylactic implantable cardioverter defibrillator was placed due to an increased risk of sudden cardiac death.

Table 1 Clinical symptoms, laboratory and echocardiographic results over time.

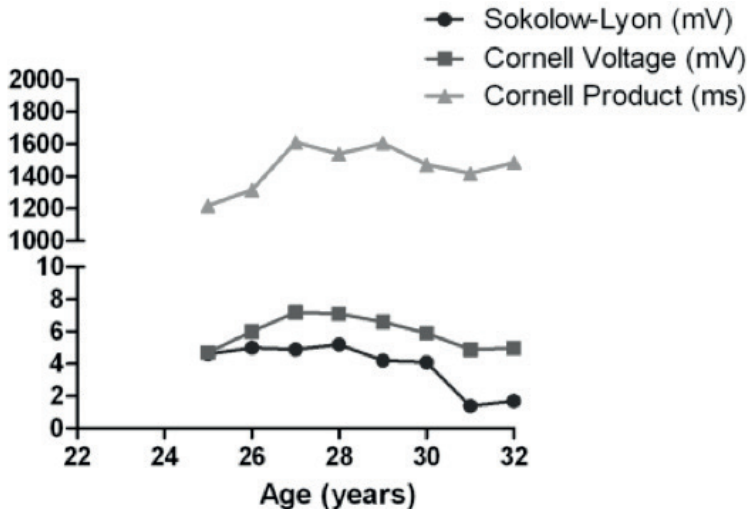
Age (years)	25	26	27	28	29	**30***	**31***	**32***
BMI !	29.7	29.7	30.9	30.3	32.2	30.5	27.7	27.8
Dyspnea §	+	+	+	++	++	-	-	-
Chest Pain §	±	±	±	+	++	-	-	-
Fatigue §	++	+	++	++	++	+	+	+
Palpitations §	-	-	-	±	+	-	-	-
NYHA classification @	2	2	2	3+	3+	2	2	2
Creatin Kinase (U/L)	578	1368	1537	3662	2712	849	1449	1400
Echocardiographic measurements (mm)								
IVS ^	22	27	32	28	32	29	22	21
PW +	18	23	24	29	25	25	25	25
LA #	34	31	33	41	42	47	47	48
LVED $	49	45	46	42	43	43	44	49
LVES %	30	34	29	27	30	28	31	33

* Bold numbers indicate data after the introduction of a high-protein, low calorie diet at the age of 30 years
! BMI indicates body mass index
§ indicates no symptoms; ± minor symptoms; + indicates intermittent symptoms; ++ indicates daily symptoms
@ NYHA classification indicates functional classification for heart failure by the New York Heart Association
^ IVS indicates interventricular septum
+ PW indicates lef ventricular posterior wall
# LA indicates left atrial dimensions
$ LVED indicates left ventricular end-diastolic dimensions
% LVES indicates left ventricular end-systolic dimensions

Due to the worsening situation the patient was evaluated for heart transplantation (HTX) at the age of 30 years. Therefore, a pre-HTX program was set up consisting of: 1. evaluation of the

status of liver and skeletal muscle, 2. a new dietary regimen to reduce weight by 10 kg, 3. peri-operative advice regarding the management of GSD III during the HTX. The new dietary regimen consisted of 24-hour protein-enriched naso-gastric drip feeding containing 900 calories per day, with protein providing 37%, carbohydrates 61%, and lipids 2% providing of total energy. After following the new dietary regimen for four months her BMI decreased to 27.7, along with a significant improvement of her complaints of constant fatigue and exercise intolerance. The NYHA classification decreased accordingly to 2 out of 4. On cardiac ultrasound concentric LVH was still present but the PW and IVS thickness had decreased to 25mm and 24mm respectively, along with the electrocardiographic LVH indices. Consequently the patient was taken off the pre-HTX program, and cardiac transplantation was deferred. The caloric intake was increased to 1370 calories per day, and the continuous naso-gastric drip feeding was stopped. The dietary regimen was switched to seven meals at daytime with two-hour intervals. Five of these meals were drinks, and two were normal meals, which were comparable in composition of energy and protein to the drinks. In the nocturnal period two gifts of cornstarch were added to reach a four-hour interval between meals. The high protein nature of the diet was maintained, and even increased to provide 43% of total energy per day compared to 37% protein per day during the pre-HTX program. After three years of follow-up she remains satisfied with reasonable exercise tolerance and minor symptoms in daily life.

Figure 1 Twelve-lead electrocardiograpy showing severe left ventricular hypertrophy with secondary repolarisation abnormalities at the age of 25, 29, 30 and 32 years old.



Discussion

We report a severely symptomatic GSDIIIa patient with cardiomyopathy who improves clinically and objectively on a high protein diet with a limited supply of carbohydrates. To our knowledge, a similar case has been reported recently in a pediatric patient (Valayannopoulos *et al* 2011), and once in an adult patient by Dagli *et al* in 2009. The latter describes a 22 year-old male with severe GSDIII related cardiomyopathy treated with a high protein diet in which overtreatment with cornstarch was avoided. Their patient improved dramatically with reversal of symptoms and echocardiographic signs of hypertrophic cardiomyopathy.

The physiology of the reversal of extreme LVH in GSDIIIa is not clear. A direct effect, where the limited supplementation of carbohydrate and increased usage of protein in gluconeogenesis reduces the cardiac storage of LD is feasible. An indirect effect is also feasible where the weight reduction reduces fat disposition in skeletal muscles, improving the condition and possible workload of the skeletal muscles and reducing the workload on the heart (Kelley *et al* 1991; Goodpaster *et al* 1999). A combination of these effects is also possible.

The majority of the patients seem to remain asymptomatic and the cardiomyopathy seems to be non-progressive (Lee *et al* 1997). Therefore, severe cases such as these emphasize the need for regular cardiac follow-up in patients with GSDIIIa related cardiomyopathy, along with the benefits of a high protein diet. In our population of GSDIII patients a high protein diet is implemented at a young age and continued through into adulthood. Through these dietary regimens GSDIII patients usually derive 20-30% of total energy from protein, which varies from four grams of protein per kilogram body weight in children and adolescents, to two grams of protein per kilogram body weight in adult patients. Therefore, renal function is also closely monitored during follow-up of these patients.

This report presents the first case of a GSDIIIa patient in whom cardiac transplantation could be deferred after initiating a low calorie, high protein diet. In younger unaffected GSDIIIa patients, this approach may prevent the development of symptomatic cardiomyopathy.

Acknowledgements

We thank Margreet van Rijn, Ph.D. and Els B. Haagsma, M.D., Ph.D. of the University Medical Centre Groningen for providing information on the natural course and dietary regimen.

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Chapter 6

Muscle ultrasound in patients with glycogen storage disease types I and III

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Ultrasound in Medicine and Biology 2016 Jan;42(1):133-42

Summary

In glycogen storage diseases (GSDs), improved longevity has resulted in the need for neuromuscular surveillance. In 12 children and 14 adults with the ‘‘hepatic’’ (GSDI) and ‘‘myopathic’’ (GSDIII) phenotypes, we cross-sectionally assessed muscle ultrasound density (MUD) and muscle force. Children with both ‘‘hepatic’’ and ‘‘myopathic’’ GSD phenotypes had elevated MUD values (MUD Z-scores: GSDI > 2.5 SD vs. GSDIII > 1 SD, $p < 0.05$) and muscle weakness (GSDI muscle force; $p < 0.05$) of myopathic distribution. In ‘‘hepatic’’ GSDI adults, MUD stabilized (GSDI adults vs. GSDI children, not significant), concurring with moderate muscle weakness (GSDI adults vs. healthy matched pairs, $p < 0.05$). In ‘‘myopathic’’ GSDIII adults, MUD increased with age (MUD-GSD III vs. age: $r = 0.71–0.83$, GSDIII adults > GSDIII children, $p < 0.05$), concurring with pronounced muscle weakness (GSDIII adults vs. GSDI adults, $p < 0.05$) of myopathic distribution. Children with ‘‘hepatic’’ and ‘‘myopathic’’ GSD phenotypes were both found to have myopathy. Myopathy stabilizes in ‘‘hepatic’’ GSDI adults, whereas it progresses in ‘‘myopathic’’ GSDIII adults. Muscle ultrasonography provides an excellent, non-invasive tool for neuromuscular surveillance per GSD phenotype.

Introduction

Glycogen storage diseases (GSD) concern a heterogeneous group of inborn errors of glycogen metabolism and/or gluconeogenesis (Dagli *et al* 2012; Kishnani *et al* 2014; Laforêt *et al* 2011). Depending on enzyme deficiency and tissue distribution, GSDs are subdivided into ''hepatic'' and ''myopathic'' phenotypes. ''Hepatic'' phenotypes are associated with an underlying glycogenolysis and/or gluconeogenesis defect, causing hepatic glycogen storage and insufficient endogenous glucose production (Laforêt *et al* 2011). Myopathic phenotypes are associated with defective muscle glycogenolysis and/or glycolysis (van Adel & Tarnopolsky 2009), causing myopathy, muscle cramps (GSDV) and respiratory insufficiency (GSDII) (van Adel & Tarnopolsky 2009).
Glycogen storage disease type I (glucose-6- phosphatase deficiency, OMIM [Online Mendelian Inheritance in Man] Nos. 232200 and 232220) concerns a ''hepatic'' phenotype with hepatic glycogen storage and non-ketotic hypoglycemia. Although glucose-6-phosphatase is not expressed in muscles, it is well known that GSDI patients may still manifest myopathic features (muscle weakness and atrophy) (Huidekoper *et al* 2010; Schwahn *et al* 2002). However, specific insight into these myopathic GSDI features is still lacking.

Glycogen storage disease type III concerns a ''mixed-myopathic'' phenotype (OMIM#23400), caused by a glycogen-debranching enzyme (GDE) gene mutation (amylo-a-1,6-glucosidase,4-a-glucanotransferase), resulting in limited dextrin storage (Yang-Feng *et al* 1992). According to differential splicing of the GDE gene, GSDIII is characterized by IIIa and IIIb subtypes. The ''myopathic'' GSDIIIa subtype is associated with progressive liver, heart and neuromuscular impairment, resulting in liver cirrhosis, hypertrophic cardiomyopathy and ambulation loss (Dagli *et al* 2012; Hobson-Webb *et al* 2010; Momoi *et al* 1992). Interestingly, the GSDIIIb subtype is characterized as a ''hepatic'' instead of ''myopathic'' phenotype (Shen & Chen 2002; Talente *et al* 1994), despite myopathic electromyography (EMG) alterations (Hobson-Webb *et al* 2010). In GSDIII patients, muscle nuclear magnetic resonance spectroscopy (NMRS) revealed that muscle weakness occurs with muscle glycogen accumulation and fat deposition (Wary et al 2010). It is still unclear whether such myopathic alterations are paralleled by elevated blood creatine kinase (CK) levels (Mogahed *et al* 2015), or not (Hobson-Webb *et al* 2010).

For improved longevity and quality of life, moderate physical exercise and clinical neuromuscular surveillance seem recommendable (Mogahed *et al* 2015; Murphy *et al* 2005; Vertilus *et al* 2010). However, insight into the progression of neuromuscular symptoms during the natural disease course is still sparse.

The non-invasive muscle ultrasound technique can detect pathologic muscle conditions, including muscle glycogen and fat deposition, by the enhanced reflection of the muscle ultrasound beam (quantified by increased muscle ultrasound density (MUD)) (Maurits *et al* 2003, 2004; Pillen *et al* 2007, 2008; Verbeek *et al* 2012, 2014). In this respect, muscle ultrasound may provide an excellent diagnostic tool in GSD patients. Because the technique is applicable to the bedside with little cooperation of the patient, it can easily be performed in young children, avoiding the necessity for more invasive procedures (such as EMG with needle electrodes, muscle biopsy and/or magnetic resonance imaging requiring anesthesia). To the best of our knowledge, muscle ultrasound studies have not been performed in GSD patients before.

In children and adults with GSDI and GSDIII subtypes, we aimed to assess and compare MUD with other neurophysiologic and clinical neuromuscular disease parameters, involving: EMG neurography, dynometry, CK values and subjective neuromuscular complaints.

Methods

Participants – The Medical Ethical Committee of the University Medical Center Groningen, The Netherlands, approved the study protocol. After informed consent by the patients and/or parents, we included 26 GSD patients (12 children [GSDI: n = 7, median age 8 y, and GSDIIIa: n = 5, median age 4 y] and 14 adults [GSDI: n = 8, median age 29 y, and GSDIII: n = 6, median age 31 y]). Because adult normative MUD and muscle force data are still incomplete, we compared each adult GSD patient with an adult control subject, resulting in 14 matched pairs (matched for age, gender and body mass). For clinical patient data involving neuromuscular complaints, CK levels and cardiac abnormalities, see Table 1.

Procedures – In GSD children and adults, we cross-sectionally determined MUD and we associated outcomes with both clinical (GSD disease type, age, neuromuscular complaints and CK levels) and neurophysiologic (muscle force and EMG–neurography with surface electrodes) parameters. All clinical and neurophysiologic assessments were performed at the University Medical Center Groningen, University of Groningen, The Netherlands.

We performed GSD muscle ultrasound recordings using General Electric Healthcare LOGIQ 9 (Jiangsu, China) equipment with a M12 L linear transducer. All recordings were obtained at standard clinical settings for muscle ultrasound: B-mode, gain (47 dB), dynamic range (DR 69), compression and time gain (neutral position). We used a linear transducer (14 MHz) and three focal points. For standardization purposes and reproducibility, time-gain compensation was preset and maintained constant during all assessments (Pillen *et al* 2006). To obtain standardized reference locations, we recorded transverse ultrasound images of the quadriceps muscle (probe placement halfway between trochanter major and lateral knee joint cleft) and tibialis anterior muscle (probe placement at one-third from the inferior part of the patella and the lateral malleolus) in supine position. We obtained transverse ultrasound images of the calf muscle (probe placement at the maximal circumference) in prone position. For muscle ultrasound assessment of the biceps muscle, we placed the probe halfway between the acromion and antecubital crease in a resting arm position (Maurits *et al* 2003).

In accordance with previously published techniques (Maurits *et al* 2003, 2004), we assessed MUD of the biceps, quadriceps, calf (gastrocnemius and/or soleus) and tibialis anterior muscles. As metabolic diseases (including GSD), are likely to affect both sides of the body symmetrically, we systematically assessed MUD on the left side of the body (in accordance with Maurits *et al* 2003). For descriptions of the applied quantification technique, see Brandsma *et al* (2012, 2014), Sival *et al* (2011) and Verbeek *et al* (2012, 2013, 2014).

In accordance with a standardized technique and protocol, we assessed muscle force in standardized positions (Beenakker *et al* 2001; van der Ploeg *et al* 1991). We determined unilateral (left) muscle force with a hand-held dynamometer (Type CT 3001, C.I.T. Technics, Groningen, Netherlands). We expressed pediatric muscle force as Z-scores of normative values (Beenakker *et al* 2001). Because dynamometry requires sufficiently reliable and active participation, dynamometry outcomes are considered as
reliable from pre-school age onward (i.e., in children older than 4 y). Because of age limitations for 4 of 5 GSDIII children included (i.e., $\leq$ 4 y) and the lack of a control value for the remaining extremely overweight child (weight-to-length ratio >+3 SD), we cannot provide reliable dynamometry data in these 6 GSDIII children.

In consideration of the incomplete adult normative muscle force data, we compared muscle force between 14 GSD and control patients (as matched pairs; matched for age, gender and body mass). We characterized the distribution of muscle force by determining summed proximal and summed distal muscle force of arms and legs. Proximal muscles include shoulder

abductors, elbow flexors, elbow extensors (arms), hip abductors, hip flexors, knee flexors and knee extensors (legs). Distal muscles include wrist extensors, three-point grip (arms) and foot dorsal-flexors (legs). Total muscle force is calculated as the summed muscle force of all proximal and distal muscles (Beenakker *et al* 2001).

Electromyography (neurography) was non-invasively performed using bipolar surface electrodes at a thermostatically controlled room temperature (Viking IV, Nicolet, Care Fusion, Houten, Netherlands). EMG–neurography parameters involved motor nerve conduction, speed and amplitude of the median, radial, ulnar and peroneal nerves, including F-responses and sensory conduction velocities of median and peroneal nerves.

Before combined presentation of neuromuscular outcome parameters in GSDIa and GSDIb patients, we checked for potentially significant differences (non-significant). As neuromuscular involvement may differ between the GSDIIIa and GSDIIIb phenotypes (see Introduction), we provide a separate neuromuscular data set for each GSDIII subgroup.

Statistical analysis – We processed data with SPSS Statistics Version 20 (IBM, Armonk, NY, USA). We expressed pediatric MUD and muscle force outcomes as Z-scores of normal values (both for individual as for summed scores) (Beenakker *et al* 2001). As muscle force and MUD data were not normally distributed (as assessed by Q–Q plots and the Shapiro–Wilk test), we compared adult GSD patients and controls using the Wilcoxon signed-rank test. We associated MUD with age and CK levels using non-parametric bivariate correlations (Kendall's t). The level of significance was set at $p < 0.05$.

Results

Glycogen storage disease type I

Clinical data – For all 15 GSDI patients (7 children and 8 adults) assessed, EMG (neurography) results were within the normal range (with respect to sensory and motor conductance velocities and amplitudes; for normal values, see Kimura [1989]). GSDI children and adults had normal blood CK concentrations and no cardiac symptoms (Table 1). Three of seven GSDI children and none of 8 GSDI adults experienced neuromuscular complaints interfering with daily life, such as (difficulties in climbing stairs, walking or swimming) (Table 1). We observed no association between elevated CK levels and neuromuscular complaints ($p = 0.94$).

MUD and muscle force in GSDI children – Children with GSDI had elevated MUD values (median Z-score in comparison with normative values: 3.3 SD, range: 0.2– 7.8 SD), with significantly higher ($p < 0.05$) MUD values in proximal than distal muscles (i.e., myopathic distribution) (Fig. 1). GSDI children had arm and leg muscle weakness (median: -0.7 SD, range: -3 to 1.8 SD), with significantly more pronounced muscle weakness in proximal than distal muscles: median of -2.3 SD (range: -3.7 to -1.6 SD) versus median of 0.4 SD (range: -1.6 to 2.4 SD), respectively. In GSDI children, we observed no association between MUD outcomes and elevated CK levels ($p = 0.70$).

MUD and muscle force in GSDI adults – Comparison of MUD between GSDI adults (median: 103, range: 67–132) and GSDI children (median: 107, range: 76–141) revealed no significant differences (Fig. 2). Comparison of MUD between GSDI adults and (age-, gender- and body mass-matched) controls revealed no significant differences, despite a tendency ($p = 0.09$) toward higher proximal MUD outcomes in GSDI adults. In GSDI adults, there was no significant association between MUD and age (data not shown). Comparison of muscle force between GSDI adults and matched controls revealed more muscle weakness in GSDI adults,

for summed-arm ($p < 0.02$), summed-proximal ($p < 0.02$) and summed-total ($p < 0.05$) muscles. Comparison of muscle weakness between GSDI adults and GSDI children revealed a similar outcome, according to a myopathic (proximal > distal, $p < 0.05$) distribution. There was no significant association between MUD and blood CK values in GSDI adults ($p = 0.54$).

Table 1 Clinical data for children and adults with GSD**.**

Patient No.	GSD type	Age	Gender	EMG	Neuromuscular complaints	Cardiac abnormalities	CK (U/L)
1	Ia	15	F	+	+[1]	None	44
2	Ia	8	F	+	-	None	86
3	Ia	16	F	+	+[2]	None	57
4	Ia	6	M	+	+[3]	None	104
5	Ia	17	F	+	-	None	71
6	Ia	8	M	+	-	None	X
7	Ib	6	F	+	-	None	51
8	IIIa	3	M	-	-	None	128
9	IIIa	2	M	-	-	None	466
10	IIIa	4	M	-	+[3]	None	355
11	IIIa	8	M	-	+[3]	CH	981
12	IIIa	4	M	-	-	None	169
13	Ia	23	F	+	-	None	85
14	Ia	26	M	+	-	None	78
15	Ia	41	F	+	-	None	27
16	Ia	31	M	+	-	None	72
17	Ia	34	M	+	-	None	56
18	Ia	26	F	+	-	None	37
19	Ia	42	F	-	-	None	87
20	Ib	18	M	+	-	None	X
21	IIIa	25	F	+	+[2, 4]	CH	1823
22	IIIa	31	F	+	+[2, 4]	CH	1093
23	IIIa	33	F	+	+[2, 4]	HOCM	1167
24	IIIa	31	F	+	+[2, 4]	CH	1332
25	IIIb	30	M	+	+[5]	None	56
26	IIIb	42	F	+	-	None	162

CH = cardiac hypertrophy (i.e., hyperplasia of the left ventricular posterior and interventricular walls); CK = creatine kinase; EMG = electromyography; GSD = glycogen storage disease; HOCM = hypertrophic cardiomyopathy (i.e., CH in association with decreased ejection fraction); - = no complaints or abnormalities; X = missing data.

[1] Impaired achievement compared with classmates.

[2] Difficulty in climbing stairs.

[3] Walking longer than 3 min.

[4] Walking longer than 20 min.

[5] Swimming longer than 15 min.

Glycogen storage disease type III

Clinical data – All seven GSDIII patients assessed (1 child and 6 adults) had normal EMG results with respect to sensory and motor conductance velocities and amplitudes. Blood CK concentrations were increased in 3 of 5 GSDIIIa children (median: 420 U/L, range: 128–981 U/L) and in 4 of 4 GSDIIIa adults (median: 1249 U/L, range: 1093–1823 U/L) (Table 1). Two of five GSDIII children and 5 of 6 GSDIII adults (GSD- IIIa: n = 4, GSDIIIb: n = 1) experienced neuromuscular complaints. Cardiac abnormalities occurred in one GSDIIIa child and in 4 GSDIIIa adults (Table 1). Elevated CK levels (.200 U/L) concurred with neuromuscular complaints in 2 of 3 GSDIII children and in all 4 GSDIII adults.

MUD and muscle force in GSDIII children – Children with GSDIII had moderately increased MUD values (median Z-scores in comparison with normative data: 1.7 SD, range: -0.9 to 3 SD), with significantly higher MUD in proximal than distal muscles ($p < 0.05$, for Z-scores of proximal muscles) (Fig. 1). Comparison of MUD values between GSDI children and GSDIII children revealed significantly higher MUD values in GSDI children for all except calf muscles: median of 110, (range: 41–131) versus median of 89 (range: 85–93), respectively ($p < 0.05$) (Fig. 3). Because of age and/or body mass limitations of all 5 GSDIII children included, we cannot provide reliable muscle force data in this group (i.e., too young).

MUD and muscle force in GSDIII adults – GSDIIIa adults had significantly higher ($p < 0.05$) MUD values than GSDIIIa children: median of 118 (range: 113–136) versus a median of 90 (range: 85–93), respectively, for all muscles assessed (Fig. 4). In GSDIIIa adults, MUD was higher in proximal than distal leg muscles: median of 118 (range: 113–144) versus median of 113 (range: 90–124) ($p = 0.046$). Comparison of MUD values between GSDIII adults and matched controls revealed higher MUD values in GSDIII adults, for all assessed leg muscles (i.e., quadriceps, tibialis anterior and calf muscles). Comparison of MUD values between 9 GSDIIIa and 2 GSDIIIb patients, suggested lower MUD values in GSDIIIb patients (Fig. 5, no statistics applicable). Comparison of MUD values between GSDIII and GSDI adults revealed significantly higher ($p < 0.05$) values in GSDIII adults for calf muscles (median of 132 [range: 101–144] vs. median of 97 [range: 83–130] and tibial anterior muscles median of 129 [range: 121–146] vs. median of 104 [range: 82–119]) (Fig. 6).

Cross-sectional MUD in GSDIII patients revealed a positive association with age (age range: 2–42 y) for biceps ($r = 0.83$), quadriceps ($r = 0.71$) and tibialis anterior ($r = 0.74$) muscles (Fig. 7). Additionally, MUD results in GSDIII patients tended to reveal a positive association with blood CK levels (median: $r = 0.42$, range: 0.16–0.49), although not statistically significant.

Adults with GSDIII had muscle weakness of the shoulder abductors, elbow extensors, finger flexors, knee extensors, summed-arm and summed-proximal muscles (all p's < 0.05). Comparison of muscle force between GSDI adults and GSDIII adults revealed more muscle weakness in GSDIII adults involving elbow extensors, summed-arm and summed-distal muscles of both arms and legs (all p were < 0.05). Comparison of muscle force between GSDIIIa ($n = 4$) and IIIb ($n = 2$) subtypes tended to reveal more muscle weakness in the former (statistical analysis not applicable because of small numbers).

Figure 1 Muscle ultrasound density (MUD) of biceps and quadriceps muscles in children with glycogen storage disease (GSD), types I and IIIa. MUD is expressed as Z-scores of normal values. In GSDI children, MUD is significantly higher than in GSDIIIa children, and MUD also exceeds normal values (Z-scores > 2.0 standard deviations (SD)).

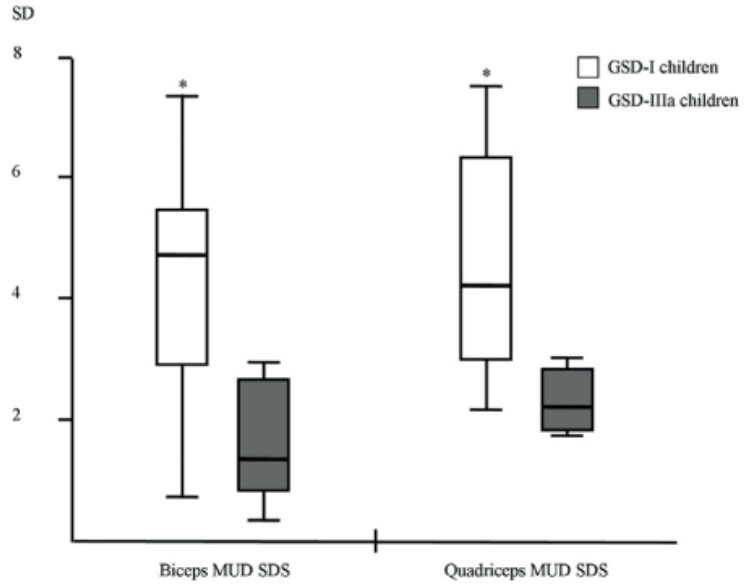


Figure 2 Muscle ultrasound density (MUD) of arm and leg muscles in patients with glycogen storage disease (GSD) type I. Comparison between children and adults revealed no significant differences.

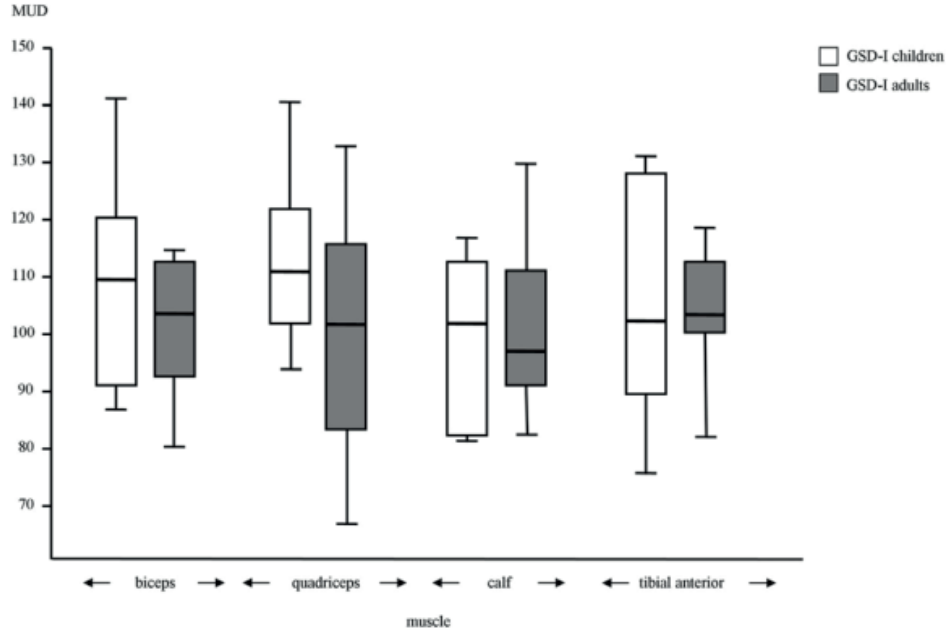


Figure 3 Muscle ultrasound density (MUD) of arm and leg muscles in children with glycogen storage disease (GSD) types I and IIIa. Children with GSDI revealed significantly higher MUD outcomes than children with GSDIIIa.

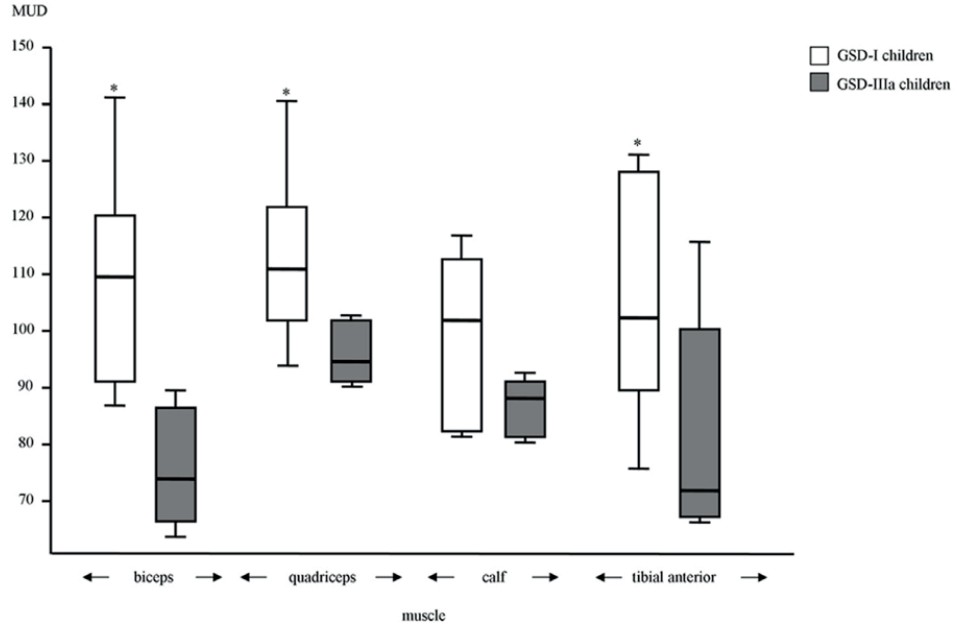


Figure 4 Muscle ultrasound density (MUD) of arm and leg muscles in patients with glycogen storage disease (GSD), type IIIa. GSDIIIa adults revealed significantly higher MUD outcomes than GSDIIIa children (for all assessed muscles).

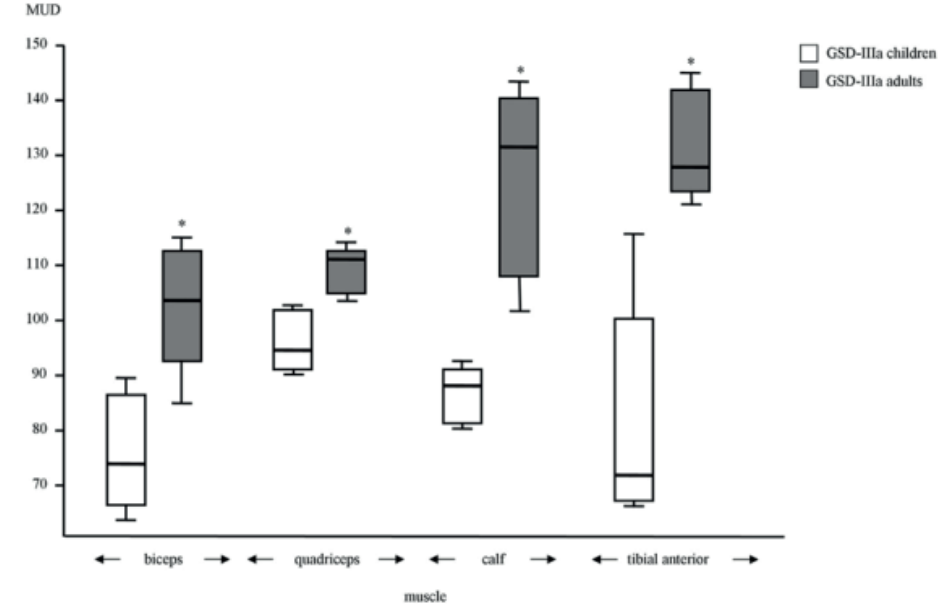


Figure 5 Muscle ultrasound density (MUD) of leg muscles in adults with glycogen storage disease (GSD) type III compared with controls. GSDIII adults revealed significantly higher MUD outcomes than age-, gender- and body mass- matched controls. MUD outcomes in two GSDIIIb patients are separately indicated (dotted line).

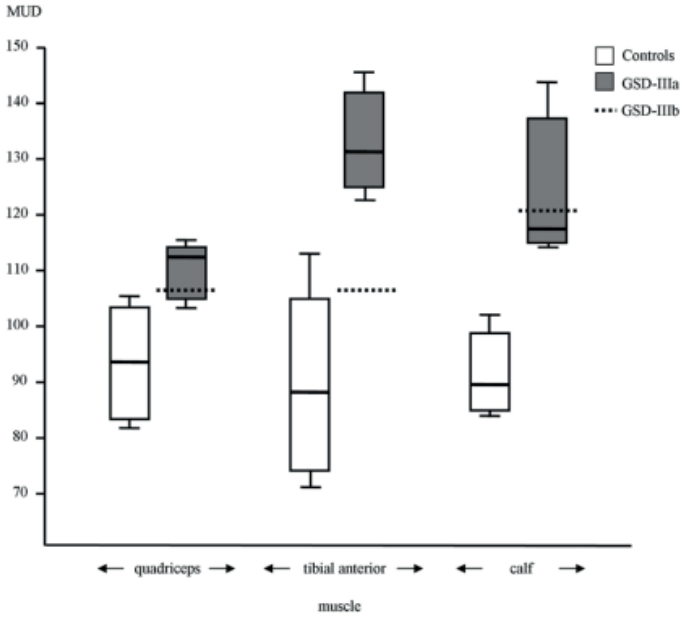


Figure 6 Muscle ultrasound density (MUD) of arm and leg muscles in adults with glycogen storage disease (GSD) types I and III. Distal leg muscles of GSDIII adults revealed significantly higher MUD outcomes than distal leg muscles of GSDI adults (distal leg muscles refer to tibialis anterior and calf muscles).

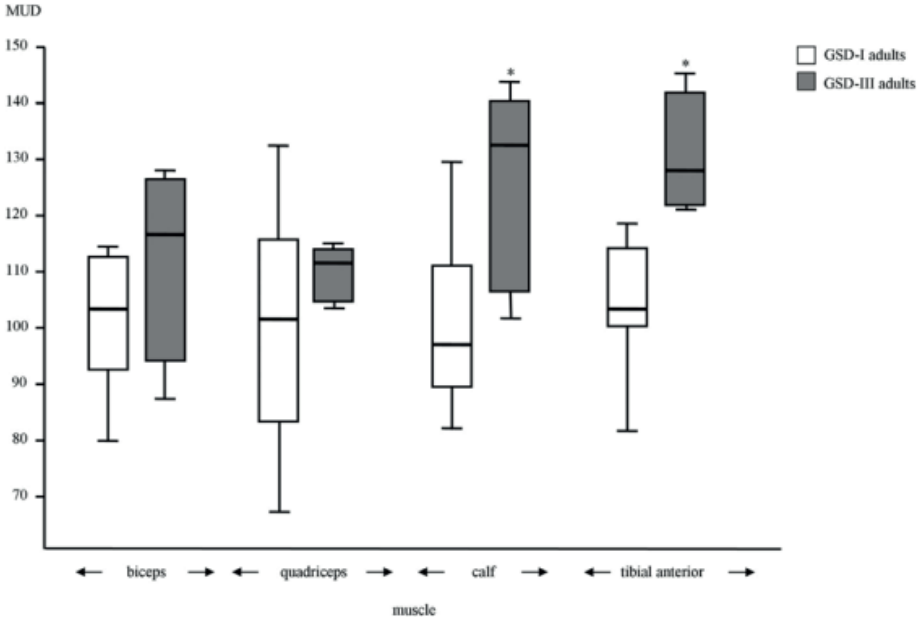
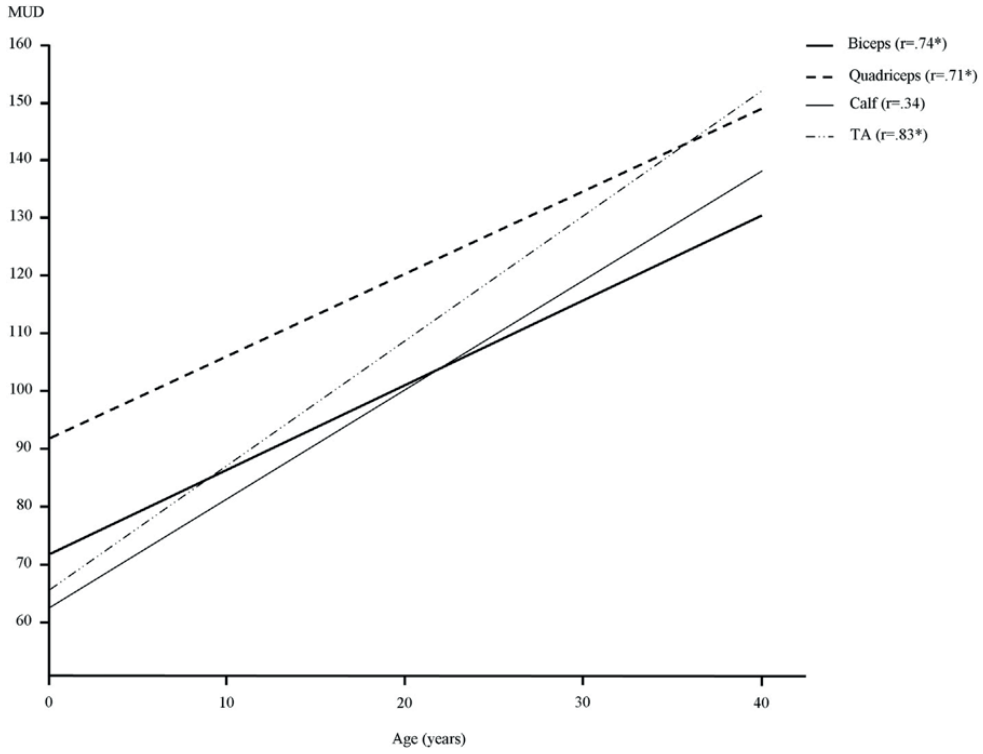


Figure 7 Relation between muscle ultrasound density (MUD) and age in patients with glycogen storage disease (GSD) type III. From child- to adulthood, GSDIII patients revealed a positive association between MUD and age (for biceps, quadriceps and tibial anterior muscles).



Discussion

In GSD patients, dietary and metabolic treatments have improved longevity, resulting in the clinical need for more consistent neuromuscular surveillance and support. In pediatric and adult ''hepatic'' and ''myopathic'' phenotypes, long-term information on neuromuscular involvement is still incomplete. In children with both ''hepatic'' and ''myopathic'' phenotypes (GSDI and GSDIII), we observed myopathy. In adult ''hepatic'' GSDI patients, myopathy stabilized, whereas in adult ''myopathic'' GSDIII patients, myopathy progressed with age.

In the assessed GSDI and GSDIII patients, we observed no neuropathic EMG alterations, as indicated by our EMG ''neurography'' data (by surface electrodes). Absent EMG ''neurography'' abnormalities are in contrast with previously published EMG ''myography'' data (obtained with invasive needle electrodes), indicating myopathy in both GSDI and GSDIII patients (Hobson-Webb *et al* 2010). From the latter perspective, we reasoned that the non-invasive muscle ultrasound technique could provide more myopathic information for each GSD subtype and age category. To the best of our knowledge, systematic muscle ultrasound data have not been obtained in GSD patients before.

In both GSDI and GSDIII children, muscle ultrasound and dynamometry data revealed myopathic involvement, with a proximal to distal (''myopathic'') distribution. Although pediatric MUD parameters appeared more strongly affected in GSDI than in GSDIII children, direct comparisons should be avoided. Especially because GSDIII patients manifested ''MUD

age-related-ness'' from childhood onward (Fig. 7) and because GSD- III children were younger than GSDI children, outcomes should also be interpreted with respect to the effect of age. Even though elevated MUD values coincided with muscle weakness, neuromuscular complaints were expressed by only a minority of the children (i.e., GSDI: 43%, GSDIII: 40%). As pediatric MUD alterations were not related to blood CK levels, we may deduce that the non-invasive muscle ultrasound technique has the potential to reveal early myopathic alterations, even in the absence of: neurographic EMG abnormalities (which are considered to be late [Kishnani *et al* 2014]), CK elevations and/or neuromuscular complaints.

In young children with the ''hepatic'' GSDI phenotype, the pathogenesis of the pronounced myopathic alterations remains unclear. Although beyond the scope of the present study, it is tempting to speculate that upregulated muscle enzyme activity could be involved. In the classic GSDI disease model, glucose production is generally considered as absent. However, stable isotope studies have indicated that these patients can have glucose production rates as high as 60% of normal (Huidekoper *et al* 2010). In GSDI patients, one might thus speculate that metabolic upregulation of muscle enzyme activity (for instance, by the debranching enzyme a-glucosidase muscle and/or glucose-6-phosphatase b [Shieh *et al* 2002]) could contribute to whole-body glucose production. In healthy patients, such endogenous glucose production has been found to decrease with age (i.e., from infancy to childhood to adulthood) (Zijlmans *et al* 2009). Analogously, one might hypothesize that myopathy in young GSDI children could reflect muscle energy deficiency during this critical period. The aforementioned myopathic alterations in children are contrasted by the ''stabilized'' neuromuscular parameters in GSDI adults. In adult GSDI patients, the ''stabilized'' neuromuscular parameters may be explained by restoration of metabolic control and decreased demands for endogenous glucose production. It is hoped future metabolic GSD studies can elucidate these potential mechanisms.

In contrast to the ''stabilized'' myopathic parameters of GSDI adults, myopathic parameters deteriorated with age in GSDIII adults (positive MUD–age relationship from childhood to adulthood). This observation appears in line with the previously described GSDIIIa disease course (Dagli *et al* 2012), gradually evolving from pediatric fasting intolerance and hypoglycemia to chronic neuromuscular pathology in adults. Although it is tempting to speculate that increased muscle glycogen and fat deposition could underlie all these age-related alterations (van Adel & Tarnopolsky 2009), other influences, such as blood vessel pathology, should also be considered (Cornelio *et al* 1984; Kiechl *et al* 1999; Wary *et al* 2010). In adult GSDIII patients, we observed concurring age-related MUD elevations, increased blood CK concentrations and other neuromuscular symptoms. These neuromuscular symptoms involved exercise intolerance, cardiac involvement and myopathic muscle weakness, with progressive extension to more muscle groups (gradually affecting the tibialis anterior too). It was recently reported that for GSDIIIa patients, the combination of underlying muscle weakness and energy deficiency is likely to result in exercise intolerance (Preisler *et al* 2013). After glucose infusion, Preisler *et al* (2013) reported improved exercise tolerance in association with increased mitochondrial fatty acid oxidation. In line with Preisler *et al* (2013), our observations suggest the potential for dietary lipid administration in patients with acute and chronic GSDIIIa disease (Derks & van Rijn 2015). In addition to the current strategy of high-protein diets (Derks & Smit 2015), it may be speculated that stimulation of fatty acid oxidation could exert a beneficial therapeutic effect by bypassing blocked carbohydrate metabolism (Derks & van Rijn 2015). It is hoped that future GSDIII research may elucidate whether dietary therapies (such as the Atkins diet [Mayorandan *et al* 2014], ketogenic diet, medium-chain triglyceride enrichment or pharmacologic agents) could have a beneficial effect on neuromuscular functioning and MUD values in GSDIII patients.

We recognize several weak points in this study. First, we obtained cross-sectional data, prohibiting delineation of individual disease trajectories. Second, because GSDI and GSDIII subtypes are relatively rare, we could only assess a limited number of patients. Third, we deliberately presented adult GSD data with reference to healthy control data (as matched pairs), because adult normal values are still incomplete.

Conclusions

In GSD, muscle ultrasonography can provide an excellent, non-invasive tool for clinical monitoring of neuromuscular pathology. In children, both ‘‘hepatic’’ and ‘‘myopathic’’ GSD phenotypes were associated with myopathic symptoms; however, myopathy stabilized in adult ‘‘hepatic’’ (GSDI) phenotypes, whereas myopathy progressed in adult ‘‘myopathic’’ (GSDIII) phenotypes. Future neuromuscular GSD studies may elucidate whether and, if so, how muscles compensate for physical demands when metabolic control is chronically altered. In consideration of the improved longevity of GSD patients, we conclude that non-invasive muscle ultrasound can provide an important contribution to neuromuscular surveillance tailored to the individual disease characteristics of each GSD patient.

Acknowledgments

We thank all the patients with glycogen storage disease, including children, parents and adults. We acknowledge all co-workers in the Department of Clinical Neurophysiology at the UMCG, especially Mrs. Carola Henzen and Mrs. Miranda Schenk. We thank Mrs. Oosterhoff and Mrs. Reiling-Wester for their administrative support.

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Chapter 7

Summary, discussion and future perspectives

The focus of this thesis was to investigate the natural course, and especially the clinical, biochemical and genetic aspects of glycogen storage disease type III (GSDIII). In GSDIII, glycogen storage is found in liver, (cardiac-) muscle, and nervous tissue (Laforêt *et al* 2012). Differences in tissue expression of the deficient GDE only partially explain the existence of various subtypes of GSDIII of which GSDIIIa (expressed in liver, skeletal muscle and sometimes heart muscle) and GSDIIIb (only expressed in liver tissue) are the most frequent subtypes (Wolfsdorf & Weinstein 2003). Due to the rarity of the disease, long-term experience with severe secondary GSDIII-related complications in different metabolic centres is limited. Until recently, no data were available on the clinical course and the associated complications of the disease. In summary, the studies in this thesis generated the following results:

Summary

Chapter 1: Provides a literature review, including a disease summary and description of hallmarks of the disease.

Chapter 2: Mutation analysis was performed in GSDIII patients using the DGGE method, which had not previously been applied on the *AGL* gene. We studied if this procedure is a reliable mutation analysis method to diagnose GSDIII. Also, we described novel *AGL* mutations among GSDIII populations. Furthermore, we studied genotype-phenotype correlation as the result of the mutation analysis may give an indication of the GSDIII subtype and clinical course.

Chapter 3: The appearance of age-related complications in the international literature, and the absence of an evidence-based consensus in dietary and pharmacological treatment, created the need for a multi-center international retrospective study (ISGSDIII) in which the natural course was investigated in a large group of patients. The aim of the ISGSDIII was to increase knowledge of diagnosis, course, and complications of GSDIII patients. Chronic complications involved the liver in 11%, cardiomyopathy in 52%, reduced exercise tolerance in 52%, muscle weakness 71%, and type 2 diabetes mellitus was diagnosed in 9% (of the adult patients).

Chapter 4: Hypertriglyceridaemia and hypercholesterolaemia were common in children under 3 years of age, with hypertriglyceridaemia correlating negatively with age. We questioned therefore, if the presence of hyperlipidaemia during childhood could put GSDIII patients at risk for early cardiovascular disease. However, no indications for an increased incidence of atherosclerosis-related complications were found in ISGSDIII (*chapter 3*).

Chapter 5: Cardiac hypertrophy and severe heart failure was reversed in an adult GSDIIIa patient by low-calorie high-protein adjustments, deferring and ultimately even preventing the need for cardiac transplantation.

Chapter 6: Progression of muscular disease in GSDIII patients is associated with increased muscle ultrasound density and decreased muscle force (with a myopathic distribution).

Discussion

Since the first description of GSDIII major progress has been made regarding clinical, biochemical, and genetic features which offers diagnostic and management options. Through dietary treatment and supportive pharmacological care morbidity and mortality because of acute metabolic derangement has decreased. With age and despite dietary management GSDIII patients may still develop chronic complications of multiple organs. The main concern of this thesis is to present a template regarding the clinical course and complications that may develop in the future.

Major points of interest are:

- Diagnosis by molecular analysis (*in chapter 2 and 3*): Most GSDIII patients have unique *AGL* genotypes, with an interesting overrepresentation of non-missense mutations are overrepresented. This is in contrast to most metabolic diseases. Currently, mutation screening methods and single-gene sequencing has been largely replaced by gene panel sequencing methods (Wang *et al* 2013; Vega *et al* 2016). Invasive diagnostic procedures such as liver biopsies have been almost completely abolished in current clinical GSD workups.
- Chronic complications (*chapter 3 and 6*): Morbidity at a later age may be very debilitating. One example is, that we observed type II diabetes in 10% of adult patients which causes paradox aims for the management of GSDIII (Oki *et al* 2000). Also, muscular involvement in GSDIIIa may cause severe morbidity which is progressive into adulthood, causing significant impairment in daily life. A similar progressive pattern for loss of muscle force in GSDIII patients was recently found in a cross-sectional retrospective single site study by Decostre *et al* in 2016. GSDIII-related cardiac complications (*chapter 5*) mostly present as cardiac hypertrophy, which may develop into cardiomyopathy. Dietary interventions can be very beneficial when metabolic control is compromised in daily life. However, when cardiac complications firstly develop in adulthood, screening for a second genetic substrate predisposing patients for cardiomyopathy may be useful (especially in consanguine families) (Dewey *et al* 2016). A correlation between the chronic complications and metabolic control (as for instance for hepatic adenomas in GSDI) could not be established.
- Hyperlipidaemia (*chapter 4*) is a common finding in young GSDIII patients, and the incidence may decrease with age and thus less variable blood glucose concentrations. Based on the ISGSDIII cohort, later in adolescent and adult life, it does not cause significant clinical cardiovascular events.

Future Perspectives

The studies presented in this thesis allow to speculate about the following future perspectives regarding some clinical hallmarks, management and follow-up:

- There is significant heterogeneity between GSD patients necessitating personalised medicine. Ideally, treatment evaluation is not limited to the hospital setting. The real world setting of the patients may even provide a better picture of treatment effectiveness and technically, telemedicine (which includes eHealth and mHealth) can integrate relevant parameters and facilitate a communication platform between patients with rare diseases and health care providers with expertise. Therefore, a *mobile GSD communication platform* is being developed to facilitate home-site metabolic monitoring and evaluation of dietary interventions by collecting biochemical, physiological and dietary follow-up parameters for individual GSD patients.

- *New treatment strategies* are warranted for both acute and chronic GSD (III) patient problems. Examples may include a modified starch (Glycosade®), dietary interventions with ketogenic diets and/or MCT-supplementation in selected cases (Valayannopoulos *et al* 2011; Brambilla *et al* 2014; Mayorandan *et al 2014*), ketone bodies (Cox *et al* 2016), gene therapy (Sun *et al* 2015), alpha-glucosidase replacement therapy (Sun *et al* 2013) and rapamycin (Yi *et al* 2014).
- Maintaining stable glucose concentrations, and thus good metabolic control, is the centre pillar of the dietary treatment of hepatic GSDs. Extended release cornstarch (Glycosade®) has been developed (Bhattacharya *et al* 2007; Correia *et al* 2008; Nalin *et al* 2015) and recently, a prospective, randomised double blind crossover clinical trial (ClinicalTrials.gov Identifier NCT02318966) has been initiated. The Glyde trial aims to establish whether Glycosade® improves short-term and long-term outcomes for patients with GSD compared to the traditional uncooked cornstarch.
- Myopathy is a common disabling phenotype in adulthood despite dietary management. Recently, acute nutritional ketosis in exercise was studied in humans and was found to enhance muscle mitochondrial function in athletes (Cox *et al* 2016). An interventional study has been started to evaluate the effect of acute ketosis on muscular performance in adult GSDIIIa patients (ClinicalTrials.gov Identifier NCT03011203).
- Glucose homeostasis in GSDIII patients remains delicate even into adulthood, development of secondary insulin resistance and insulin-dependent diabetes mellitus in older GSD III patients may complicate treatment even more (Dewey *et al* 2016; Lee *et al* 1997). Related to this, lipid metabolism seems to be perturbed as well, particularly in younger patients. In this perspective, the development of diabetes mellitus type II in 10% of the GSD III patients could be viewed in the light of the metabolic syndrome.
- *Animal models* have been proven to be very useful in research on disease pathophysiology and the testing of novel therapeutic options. Two GSDIIIa animal models have been developed in recent years, a canine (Yi *et al* 2012; Brooks *et al* 2016) and mouse model (Liu *et al* 2014; Pagliarani *et al* 2014). The canine model of GSDIII has a homozygous frameshift mutation in exon 32 in *AGL* leading to the deletion of 126 amino acids, causing a GSDIIIa phenotype which was confirmed by enzyme activity measurements in muscle and liver tissue. Biochemically, normolipidaemia was observed in this model in contrast to human GSDIIIa, liver- and muscle enzymes however (AST, ALT, AP and CK) were elevated (Yi *et al* 2012). In follow-up, these values decreased and the dogs showed liver and muscle fibrosis, matching human GSDIIIa (Brooks *et al* 2016). Two mouse models have been developed, one by removing the exons after exon 5 in *AGL*, the other by the deletion of exons 32-34 (Liu *et al* 2014; Pagliarani *et al* 2014). These models showed severe glycogen accumulation in liver and muscle tissue, even in the central nervous system and the diaphragm. Also in these models, normolipidaemia was observed. While both models have most of the hallmark features of GSDIIIa, only the canine model has been used for testing experimental therapeutic options. In the canine GSDIIIa model rapamycin (an inhibitor of mTOR) has been shown to reduce glycogen accumulation in muscle cells and prevent hepatic fibrosis suggesting therapeutic possibilities for GSDIIIa patients (Yi *et al* 2014).
- There is a need for collaborative *international patient registries* for rare diseases, including hepatic glycogen storage diseases (like GSDIII). To study and answer remaining questions regarding certain clinical features and dietary/pharmacological treatment in the future, a prospective database is warranted which focusses on hepatic GSDs in general with the support of a global network of treatment centres.

- The recent development and national endorsement of expertise centres for very rare diseases (such as hepatic glycogen storage diseases) and the construction of formal international networks (such as the European Reference Network for Hereditary Metabolic Diseases (MetabERN)) may promote the development of evidence based treatment guidelines, patient empowerment and governance structures.
- There is a need to structure and prioritise *future research agendas*. In the last decade, the James Lind Alliance (www.jla.nihr.ac.uk) has facilitated Priority Setting Partnerships (PSPs) for several medical conditions (Partridge *et al* 2004). To date these PSPs have focussed on common disorders and they have been organised on a national level. This year a PSP will be created to formulate the Top 10 priorities for future research for children and adults with hepatic GSD. This will be the first international, multilingual PSP focussing on ultra-rare inherited metabolic diseases. It is expected that the outcome will promote patient empowerment in scientific research and that research topics that are most important to the patients may be addressed sooner and more effectively.

In conclusion, ISGSDIII and the other studies in this thesis have contributed to an expansion of the knowledge on the clinical course of GSDIII and its related complications. Also, the identification of lapses in knowledge through ISGSDIII have given room for the development of novel studies and initiatives to fill these gaps. Therefore, monogenetic GSDs already have, and will continue to serve as models for prevalent, multifactorial disorders of carbohydrate and lipid metabolism.

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Nederlandse samenvatting

Abbreviations

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Curriculum vitae

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Nederlandse samenvatting

Dit proefschrift had als doel om het natuurlijk beloop te belichtten van glycogeenstapelingsziekte type III (GSDIII), en dan vooral de klinische, biochemische en genetische aspecten. Bij GSDIII-patiënten stapelt zich glycogeen in de lever, (hart-) spierweefsel, en zenuwweefsel. Verschillen in de weefselexpressie zijn echter slechts een gedeeltelijke verklaring voor het bestaan van verschillende subtypes van GSDIII. Hiervan zijn GSDIIIa (wat tot uitdrukking komt in de lever, skeletspieren en soms het hart) en GSDIIIb (wat alleen tot uitdrukking komt in de lever) de meest voorkomende subtypes. Omdat GSDIII een zeer zeldzame ziekte is, is er weinig lange-termijn ervaring met de secundaire complicaties – zelfs in grote metabole centra. Tot voor kort waren er tevens slechts weinig wetenschappelijke data beschikbaar met betrekking tot het natuurlijk beloop en deze secundaire complicaties. In dit proefschrift hebben we specifiek naar het beloop en deze complicaties gekeken, en samengevat zijn dit de resultaten:

- *Hoofdstuk 1* is een samenvatting van de bekende literatuur, waarbij een samenvatting wordt gegeven van de belangrijkste kenmerken van het ziektebeeld.

- In *hoofdstuk 2* presenteren we een mutatieanalyse methode (DGGE) voor het *AGL*-gen, die voorheen nog niet was toegepast. We hebben deze methode toegepast in een populatie GSDIII-patiënten, en hebben onderzocht of DGGE betrouwbaar is om het *AGL*-gen mee te analyseren. Tevens worden in dit hoofdstuk nieuwe mutaties beschreven, en hebben we gekeken of er een relatie is tussen de gevonden mutaties (inclusief de reeds bekende mutaties) en het klinische fenotype.

- *Hoofdstuk 3* beschrijft de resultaten van een multicenter internationaal retrospectief onderzoek naar het natuurlijk beloop van GSDIII. Dit onderzoek werd gedaan in de (tot nu toe) grootste groep GSDIII-patiënten, en had als doel om de kennis over de diagnosestelling, het beloop, en de complicaties uit te breiden. De meest frequente complicaties betroffen de lever (bij 11% van de patiënten), cardiomyopathie (52%), lage inspanningstolerantie (52%), spierzwakte (71%), en diabetes mellitus type 2 (9%, van de volwassen patiënten).

- In *hoofdstuk 4* gaan we nader in op de hypertriglyceridemie en hypercholesterolemie die veel voorkomt bij GSDIII-patiënten, vooral bij kinderen onder de 3 jaar. Hierbij correleerde hypertriglyceridemie negatief met leeftijd. Om deze reden kwam de vraag naar voren of hyperlipidemie op jonge leeftijd het risico op hart- en vaatziekten op latere leeftijd verhoogde. Er werden echter in *hoofdstuk 3* geen aanwijzingen gevonden dat artherosclerose bij volwassen GSDIII-patiënten een veelvoorkomende complicatie is.

- In *hoofdstuk 5* beschrijven we de casus van een volwassen GSDIIIa-patiënt die een ernstige hypertrofische cardiomyopathie ontwikkelde en daardoor in aanmerking kwam voor een harttransplantatie. De cardiomyopathie kon echter succesvol behandeld worden met een calorieën-beperkt eiwitrijk dieet, waardoor de hypertrofie terugliep en een harttransplantatie niet nodig was.

- De focus in *hoofdstuk 6* ligt op de skeletspieren waarbij we middels het meten van de echogeniteit van de spieren en het doen van krachtmetingen al op jonge leeftijd myopatische veranderingen bij GSDIII-patiënten konden vaststellen.

Sinds GSDIII voor het eerst werd vastgesteld in het midden van de vorige eeuw is er een enorme vooruitgang geboekt wat betreft de diagnostiek, behandeling, en het monitoren van de effectiviteit van de behandeling. Dit proefschrift dient als een blauwdruk voor het beloop van GSDIII in een grote groep patiënten, iets wat nog nooit eerder is gedaan. Op basis van deze blauwdruk kan een duidelijker toekomstbeeld gegeven worden aan patiënten en behandelaars.

Abbreviations

ALAT	alanine aminotransferase
ASAT	aspartate aminotransferase
BMD	bone mineral density
BMI	body mass index
CGDF	continuous gastric drip feeding
CK	creatine kinase
CRF	case record form
CSCE	conformation sensitive capillary electrophoresis
CVD	cardiovascular disease
DGGE	denaturing gradient gel electrophoresis
DM2	type 2 diabetes mellitus
EMG	electromyography
ESGSDI	european study on glycogen storage disease type I
ESP	exome sequencing project
GDE	glycogen debranching enzyme
GSD	glycogen storage diseases
GSDI	glycogen storage disease type I
GSDIII	glycogen storage disease type III
HCC	hepatocellular carcinoma
HTX	heart transplantation
ISGSDIII	international study on glycogen storage disease type III
IVS	intraventricular septum
LD	limit dextrin
LVH	left ventricular hypertrophy
MUD	muscle ultrasound density
NYHA	new york heart association
PSP	priority setting partnership
PW	posterior wall
UCCS	uncooked cornstarch

Dankwoord

Dit proefschrift is grotendeels het resultaat van de samenwerking met collega's uit binnen- en buitenland, en ik denk dat tijdens die periode mijn dankbaarheid en enthousiasme al tot uiting zijn gekomen. Onderzoek zoals in dit proefschrift beschreven is echter alleen mogelijk dankzij de patiënten (en de ouders), en ik wil hen daarom als eerste bedanken.

De mensen die het onderzoek mogelijk hebben gemaakt wil ik als volgende bedanken. Mijn promotor Peter en co-promotor Terry, zonder jullie was dit project nooit tot stand gekomen en was het onmogelijk geweest het te completteren. Peter, bedankt voor de ingang die je me gaf voor dit onderzoek als wetenschapsstage, en voor het vertrouwen dit uit te mogen bouwen tot een promotieproject. De jaren dat ik in het UMCG doorbracht was je altijd beschikbaar voor vragen en overleg, en met jouw contacten kon ISGSDIII van de grond komen. Terry, bedankt voor je advies en motivatie. De laatste periode ben je een enorme drijfveer geweest achter dit project en achter mijn promotie. Met jouw geduld en inzicht werd dit tot een succes.

Without the support, guidance and hospitality of many colleagues from around the globe ISGSDIII would not have been possible. Thank you “ISGSDIII team”! I would especially like to thank Prof. dr. David Weinstein, who mentored me during my time in Florida and was a driving force behind ISGSDIII. You are an amazing example of what one person can do when he puts his mind to it and your dedication to progress in the treatment of hepatic GSD will prove to be essential to the success of new therapeutic strategies. I also owe a lot of gratitude to Dr. Ulrike Steuerwald and Dr. Elaine Murphy for their hospitality and help during my time in Tórshavn and London, respectively. I would like to thank the members of the assessment committee, Professor dr. Reijngoud, Professor dr. van der Ploeg and Professor dr. Santer for their assessment.

Mijn kamergenoten Michelle, Jozien en Janyte: we hebben slechts een jaar samen in de kelder van het Triadegebouw doorgebracht maar het was een onvergetelijke tijd. Soms waren er lastige dagen maar dankzij jullie ondersteuning en humor was het een "year to remember"!

Dan de paranimfen – Egbert, we hebben elkaar de afgelopen jaren niet veel gezien, en met onze drukke banen zal dat lastig blijven. Desondanks ben je altijd een van mijn beste vrienden gebleven en is het elke keer dat we elkaar spreken meteen weer als vanouds. Dank dat je er altijd bent geweest en gebleven.

Lieve Marleen, jij bent het meest fantastische wat mij ooit is overkomen en degene met wie ik altijd samen wil zijn. In mijn promotieperiode zijn we elkaar lang uit het oog verloren, maar niet uit het hart. Gelukkig is dat in het afgelopen jaar veranderd, en ik weet dat we nog zoveel mooie dingen voor de boeg hebben.

Lieve Sjieuwke Lies en Hessel Jan, jullie zijn over de jaren mijn beste vrienden geworden en maken mij altijd aan het lachen, dankzij jullie is het glas altijd halfvol. Lieve papa en mama, bedankt dat jullie altijd achter me staan en voor jullie grenzeloze vertrouwen. "Je kan het!" kreeg ik altijd weer te horen en dat heeft me gebracht waar ik nu ben. Dankzij dit mantra en de ondersteuning die daaruit voortkomt kan ik mijn dromen verwezenlijken.

Curriculum Vitae

Christiaan Peter Sentner was born on September 20, 1984 in Groningen, and was raised in Buitenpost, the Netherlands. On elementary school *De Fakkel* the groundwork was laid for starting pre-university school (Atheneum) at the *Lauwers College* in 1996. He graduated in 2002 and started studying medicine at the University of Groningen. During this time, he completed a scientific internship on glycogen storage disease type III at the department of metabolic diseases under the supervision of Prof. dr. G.P.A. Smit. In 2008, this internship resulted in the application and admission to the MD/PhD programme which resulted in the publications presented in this thesis (also under the supervision of Dr. T.G.J. Derks). After completing his medical internships and graduating in 2011 and an additional full year of scientific research in 2012, he started his residency in paediatrics in 2013 in Oldenburg, Germany (under the supervision of Prof. dr. C. Korenke, Prof. dr. H. Müller and Prof. dr. J. Seidenberg). He will complete his residency in 2018 and then start his fellowship in the paediatric intensive care unit.

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